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Study of ethylene signalling in *Arabidopsis thaliana* by transcript profiling and mutant analysis

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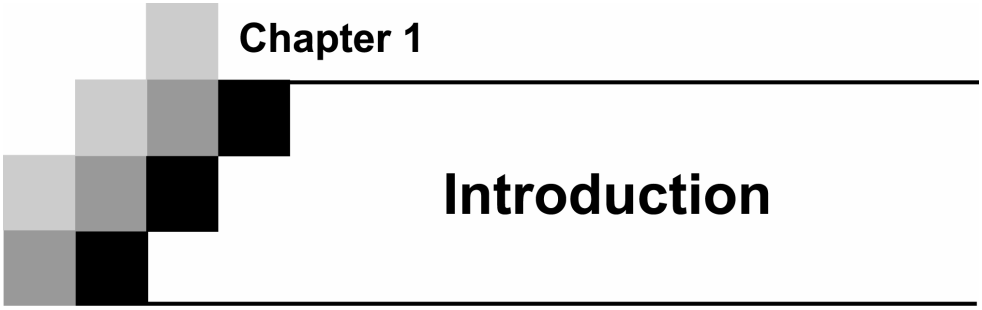
List of abbreviations

ABA	abscisic acid
ABC transporter	ATP-binding cassette transporter
abi	ABA insensitive
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
AFLP	amplified fragment length polymorphism
AGL	AGAMOUS-like
AGP	arabinogalactan protein
AIB	aminoisobutyric acid
ain	ACC insensitive
alh	ACC-related long hypocotyl
ANOVA	analysis of variance
AP	apetala
AQBC	adaptive quality-based clustering
arc	accumulation and replication of chloroplasts
ATP	adenosine triphosphate
aux	auxin resistant
AVG	aminoethoxyvinylglycine
bp	base pair
CAB	chlorophyll A/B-binding protein
CATMA	complete Arabidopsis transcriptome microarray
cDNA	complementary DNA
ch	chlorina
cin	cytokinin insensitive
ckh	cytokine hypersensitive
cM	centiMorgan
Col-0	Columbia-0
ctr	constitutive triple response
DNA	deoxyribonucleic acid
EBF	EIN3-binding F box protein
EDF	ethylene response DNA-binding factor
EDTA	ethylenediaminetetraacetic acid

eer	enhanced ethylene response
eil	ein-like
ein	ethylene insensitive
EMS	ethylmethanesulfonate
era	enhanced response to ABA
EREBP	ethylene response element binding factor protein
ERD	early-responsive to dehydradation
ERF	ethylene responsive factor
ers	ethylene response sensor
eto	ethylene overproducer
etr	ethylene resistant
EXP	expansin
FN	fast neutrons
GA	gibberrilin acid
gin	glucose-insensitive
glo	glucose-oversensitive
GM	germination medium
GST	glutathione-S-transferase
HLH	helix-loop-helix
hls	hookless
HPK	histidine protein kinase
HSP	heat shock protein
IAA	indole-3-acetic acid
JA	jasmonic acid
Ler	Landsberg *erecta*
LNM	low nutrient medium
MAPK	mitogen-activated protein kinase
MCP	methylcyclopropene
MIPS	Munich Information Center for Protein Sequences
MMK	Medicago MAP kinase
mRNA	messenger RNA
MTA	methylthioadenosine
NPA	1-*N*-naphtylphthalamic acid
NR	nitrate reductase
PERE	primary ethylene responsive element

PCR	polymerase chain reaction
PR	pathogenesis related
ran	responsive to antagonist
RAP	related to AP
RAV	related to ABI3/VP1
RBP	RNA binding protein
RD	responsive to dehydratation
REML	Restricted maximum likelihood
RNA	ribonucleic acid
ROS	reactive oxygen species
SAM	S-adenosylmethionine
SCF	Skp1, Cullin, F-box-class protein
SIMK	salt stress-induced MAPK
SIMKK	SIMK kinase
slo	slow
SSLP	single sequence length polymorphism
TCO	trans-cyclo-octene
TIBA	tri-iodo-benzoic acid
TP	transcript profiling
UBC	ubiquitin conjugating enzyme
UBP	ubiquitin specific protease
UBQ	ubiquitin
wei	weak ethylene insensitive
XET	xyloglucan endotransglycosylase



Chapter 1

Introduction

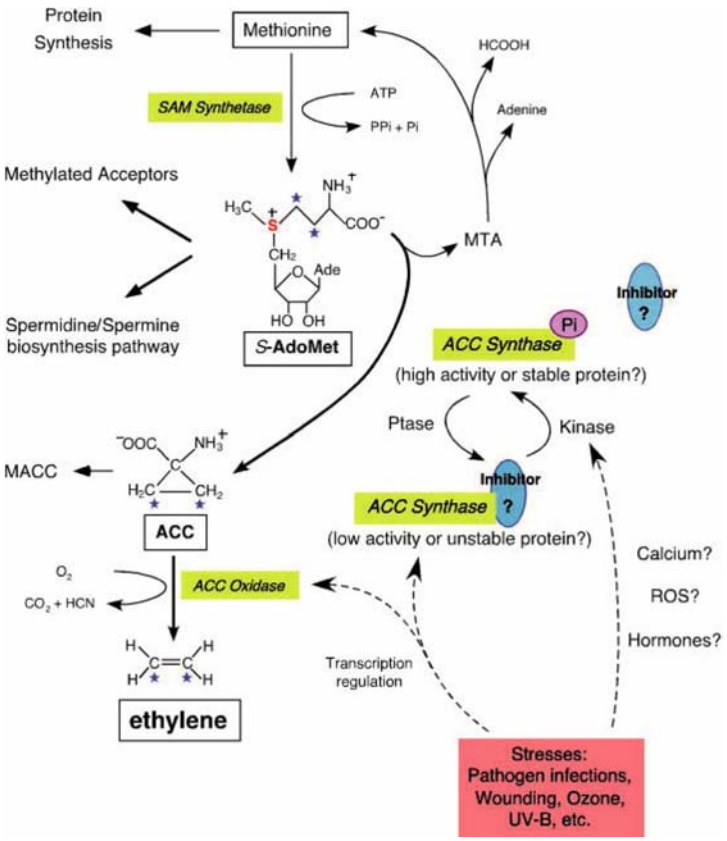
Chapter 1: Introduction

Ethylene (C_2H_4) is a simple gaseous hydrocarbon that has profound effects on plant growth and development (Abeles et al., 1992). Ethylene was one of the first plant hormones discovered. Indeed, in the nineteenth and early twentieth centuries, illuminating gas produced from coal was used for lightning. Leaks from pipelines carrying illuminating gas resulted in premature senescence and abscission in nearby vegetation. Dimitri Neljubov identified ethylene as the active component of illuminating gas and published his results in 1901. In the 1930s, plants were demonstrated to produce ethylene themselves. From that time on, ethylene was established as an endogenous regulator of plant growth and development. Ethylene is involved in many aspects of the life cycle, including seed germination, root hair development, seedling growth, leaf and petal abscission, fruit ripening and organ senescence (Yang and Hoffman, 1984). The production of ethylene is tightly regulated by internal signals during development and in response to environmental stimuli from biotic (e.g., pathogen attack) and abiotic stresses, such as wounding, hypoxia, ozone, chilling or freezing. To understand the functions of ethylene in plant growth, it is important to know how this hormone is synthesized, how its production is regulated, and how the signal is transduced.

1.1 BIOSYNTHESIS OF ETHYLENE: MECHANISMS AND REGULATION

Almost all plant tissues have the capacity to make ethylene, although in most cases the amount of ethylene production is very low. The ethylene biosynthetic pathway was elucidated in a series of elegant studies, principally by Yang and co-workers (Yang and Hoffman, 1984; Kende, 1993). Ethylene is derived from the amino acid methionine, which in the first step is converted to *S*-adenosyl-methionine (*S*-Adomet) by *S*-Adomet synthetase (SAM synthetase) (Fig.1). *S*-Adomet is the major methyl donor in plants and is used as a substrate for a number of biochemical pathways, including polyamines and ethylene biosynthesis (Ravanel et al., 1998). In addition, *S*-Adomet is involved in methylation reactions that modify lipids, proteins, and nucleic acids. ACC synthase (ACS), which converts *S*-Adomet to 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang and Hoffman, 1984), is the first committed and generally rate-limiting step in ethylene biosynthesis. In addition to ACC, ACC synthase also produces 5'-methylthioadenosine (MTA), which is then converted to methionine by using a modified methionine cycle (Bleecker and Kende, 2000). This salvage pathway preserves the methyl group for another

round of ethylene production. Therefore, ethylene can be synthesized continuously without demanding an increasing pool of methionine. The observations that active ACC synthase is labile and present at low levels suggest that ethylene biosynthesis is tightly controlled. Finally, ACC is oxidized by ACC oxidase to form ethylene, CO_2, and cyanide, which is detoxified to β-cyanoalanine by β-cyanoalanine synthase to prevent toxicity of accumulated cyanide during high rates of ethylene biosynthesis.



From Wang et al., 2002

Figure 1: Biosynthetic pathway and regulation of ethylene. The formation of *S*-AdoMet is catalyzed by SAM synthetase from the methionine at the expense of one molecule of ATP per molecule of *S*-AdoMet synthesized. The rate-limiting step of ethylene biosynthesis is the conversion of *S*-AdoMet to ACC by ACC synthase. ACC is the immediate precursor of ethylene. MTA is the by-product generated along with ACC production by ACC synthase. Malonylation of ACC to malonyl-ACC (MACC) deprives the ACC pool and reduces the ethylene production. ACC oxidase catalyses the final step of ethylene synthesis using ACC as substrate and generates carbon dioxide and cyanide. Transcriptional regulation of both ACC synthase and ACC oxidase is indicated by dashed arrows.

1.1.1 ACC synthase: A multigene family in plants

ACC synthase is encoded by a multigene family whose structure resembles the subgroup I family of pyridoxal 5'-phosphate (PLP)-dependent aminotransferases (Mehta et al., 1993). The *Arabidopsis* genome contains twelve annotated ACC synthase genes *(ACS1-12)*; however the *ASC3* gene is most likely a pseudogene and *ACS1* encodes a non-functional

ACC synthase (Liang et al., 1995; Liang et al., 1996; Yamagami et al., 2003). This is similar to other plant species, such as tomato and rice, in which ACS is encoded by a multigene family. In *Arabidopsis*, *ACS1* and *ACS3* do not encode for active ACS as was shown by expression of these genes in either bacterial or yeast expression systems (Liang et al., 1995). ACS1 is missing a highly conserved tripeptide, which is located near the active site and may be essential for ACS activity (Liang et al., 1995). Deletion of this tripeptide from ACS2 inactivates it. On the other hand, *ACS3* is believed to be a pseudogene resulting from a partial duplication of *ACS1*. It is possible that ACS1 may function as a regulator of ACS activity through dimerization with other ACS enzymes since it is induced by several signals that activate other ACS genes (Wang et al., 2002). Inhibition of protein synthesis by cycloheximide treatment induces expression of several ACS genes, suggesting that these genes are under negative control by a short-lived transcriptional repressor, or that these transcripts are degraded by a short-lived nuclease (Liang et al., 1992). An alternate explanation is that cycloheximide treatment results in retention of mRNA on the ribosomes; therefore, the steady state level of ACS mRNA is relatively increased.

A diverse group of factors have been described as modulators of the level of ethylene biosynthesis in numerous plant species. In *Arabidopsis*, these inducers include auxin, cytokinin, brassinosteroids, ethylene, ozone, copper, mechanostimuli, pathogens and wounding (Van der Straeten et al., 1992; Rodrigues-Pousada et al., 1993; Botella et al., 1995; Cary et al., 1995; Liang et al., 1996; Vahala et al., 1998; Woeste et al., 1999). As in other species, the *Arabidopsis* ACS genes are differentially regulated by these factors. Previously, seven *Arabidopsis* ACS genes have been characterized (Liang et al., 1992; Van der Straeten et al., 1992; Arteca and Arteca, 1999; Samach et al., 2000). *ACS2* is induced by cycloheximide, wounding, and 2h of ethylene treatment. The ethylene-induced expression gradually decreases with prolonged ethylene exposure, suggesting negative feedback regulation of *ACS2* (Van der Straeten et al., 1992; Liang et al., 1996). Moreover, ACS2 plays an important role in vegetative development including lateral root formation (Rodrigues-Pousada et al., 1993). The *ACS4* gene has been shown to be a primary auxin response gene with auxin-responsive motifs present in the 5' upstream region of this gene (Abel et al., 1995). Furthermore, *ACS4* is induced in seedlings by cycloheximide and wounding (Liang et al., 1992). *ACS5* is induced by lithium chloride and low concentration of cytokinin only in etiolated seedlings (Liang et al., 1996; Vogel et al., 1998b). *ACS6* can be induced specifically by cyanide treatment, exposure to ozone in light-grown leaves and mechanical strain by touching; but also by cycloheximide, indoleacetic acid (IAA), and

ethylene (Vahala et al., 1998; Overmyer et al., 2000; Smith and Arteca, 2000). *ACS10* was identified as one of the early targets of *CONSTANS*, which promotes flowering of *Arabidopsis* in response to light (Samach et al., 2000). Recently, Yamagami et al. (2003) further demonstrated that 2h treatment with IAA induces all of the active *ACS* genes, except *ACS7* and *ACS9*. Their results further indicated that the *Arabidopsis* ACS isozymes are biochemically distinct. It is proposed that biochemically diverse ACS isozymes function in unique cellular environments for the biosynthesis of ethylene, permitting the signalling molecule to exert its unique effects in a tissue-or cell-specific fashion.

1.1.2 Regulation of ACS: Post-transcriptional regulation

Genetic analysis of the regulation of ethylene biosynthesis in *Arabidopsis* has provided compelling evidence that ACC synthase can also be regulated post-transcriptionally (Vogel et al., 1998b; Woeste et al., 1999). Three ethylene overproduction mutants *eto1*, *eto2*, and *eto3* have been identified in *Arabidopsis* (Guzman and Ecker, 1990; Kieber et al., 1993). *eto1* is a recessive mutation, whereas *eto2* and *eto3* are dominant. The constitutive triple-response phenotype of the *eto* mutants can be suppressed by silver thiosulfate (inhibitor of ethylene perception) or aminoethoxyvinylglycine (AVG) (inhibitor of ethylene biosynthesis), suggesting that these mutants are affected in the regulation of ethylene biosynthesis. Molecular analysis revealed that the *eto2* mutation was the result of a single nucleotide insertion that disrupted the C-terminal 11 amino acids of ACS5 (Vogel et al., 1998b). Although the steady state *eto2* mRNA shows little change, ethylene overproduction in *eto2* etiolated seedlings is 20-fold that of the wild-type, suggesting that the increased activity is not the result of gene expression (Vogel et al., 1998b). Further evidence that *ACS5* is post-transcriptionally regulated came from the analysis of the *cin*-mutants (Vogel et al., 1998). It has been shown that low doses of cytokinin (0.5 to 10 μM) stimulate ethylene production in etiolated seedlings of *Arabidopsis* and induce morphological changes resembling the triple response upon ethylene treatment in darkness (Cary et al., 1995). This has been exploited to identify mutants that fail to increase ethylene in response to cytokinin (Vogel et al., 1998, 1998a). Five complementation groups, termed cytokinin-insensitive mutants (*cin1* to *cin5*), have been found. Recessive mutations in one of these complementation groups, *cin5*, were mapped very close to *eto2* and *ACS5*. *CIN5* was subsequently found to correspond to *ACS5*, suggesting that this isoform is the major target for cytokinin regulation (Vogel et al., 1998b). In addition, cytokinin-mediated ethylene production does not correlate with an induction of *ACS5* mRNA, demonstrating that

cytokinin increases ACS5 function primarily by a post-transcriptional mechanism. Together, these results indicate that the C-terminus of *ACS5* negatively regulates the function of the protein, and cytokinin may elevate ethylene biosynthesis by partially relieving this inhibition. Indeed, Chae et al. recently described that the analysis of purified recombinant ACS5 and epitope-tagged ACS5 in transgenic *Arabidopsis* revealed that *eto2* does not increase the specific activity of the enzyme either in vitro or in vivo; rather, it increases the half-life of the protein (Chae et al., 2003). In a similar manner, cytokinin treatment increased the stability of ACS5 by a mechanism that is at least partially independent of the *eto2* mutation. Previous results in tomato have demonstrated that Le-ACS2 is phosphorylated on a serine residue present in the carboxy terminus (Tatsuki and Mori, 2001); this serine residue is conserved in ACS5 and is disrupted by the *eto2* mutation. Based on the presence of the highly conserved serine residue in ACS enzymes from many different species, negative regulation may represent a general mechanism to modulate very rapid changes in ACS activity, without the requirement to modulate *ACS* gene transcription. Like *eto2*, the *eto1*- and *eto3*- mutations were likely to affect the post-transcriptional regulation of ACS function. The *ETO1* gene has recently been cloned (Cosgrove et al., 2000). It encodes a protein containing putative peptide binding domains and interacts with the wild type, but not the *eto2* version of the ACS5 isoform in the yeast two-hybrid system. Furthermore, ETO1 can inhibit the activity of ACS5 in vitro. The *eto1* mutation was found to act by increasing ACS5 function by stabilizing the ACS5 protein (Chae et al., 2003). Furthermore, the *eto3* phenotype is the result of a missense mutation within the C-terminal domain of ACS9, similar to the *eto2* mutation (Chae et al., 2003). These results suggest that an important mechanism by which ethylene biosynthesis is controlled is the regulation of the stability of ACS proteins, mediated at least in part through the C-terminal domain.

1.1.3 ACC-oxidase-family

The final step in ethylene biosynthesis, the conversion of ACC to ethylene, is catalyzed by the enzyme ACC oxidase (ACO), which was previously called ethylene-forming enzyme. ACC oxidase may play an important role in regulating ethylene biosynthesis, especially during conditions of high ethylene production which include pollination-induced senescence, wounding, and senescence of leaves, fruit and flowers (Nadeau et al., 1993; Kim and Yang, 1994; Barry et al., 1996; Lasserre et al., 1996; Tang and Woodson, 1996). The ACC oxidase gene has been cloned from many different plant species. In *Arabidopsis*, ACO is present as a multigene family, but little information about these genes has been

reported (Gomez-Lim et al., 1993; Raz and Ecker, 1999). An ACO gene, *AtACO2*, has been shown to be expressed preferentially on the outer cells of the apical hook in etiolated *Arabidopsis* seedlings and may play a role in regulating the differential cell elongation that is responsible for the formation of this structure (Raz and Ecker, 1999). The steady-state level of the *AtACO2* transcripts increased in response to exogenous ethylene (Kieber et al., 1993; Raz and Ecker, 1999).

With the completion of the *Arabidopsis* genome sequence, a number of new ACS and ACO homologs have been identified, providing input for future studies. This raises the question of why plants require multigene families for ACS and ACO and whether these proteins have equivalent biochemical activities and regulation. Regulatory molecules will likely include transcription factors that activate or modulate gene expression as well as enzymes such as kinases and phosphatases that may post-transcriptionally modulate activity.

1.2 ETHYLENE SIGNALLING

After its synthesis, ethylene is perceived by a family of receptors and its signal transduced through the transduction machinery to trigger specific biological responses. Mutant isolation in *Arabidopsis* has relied almost exclusively upon one mutant screen: the triple response. Dark-grown seedlings exhibit several phenotypic responses to ethylene that are collectively termed the triple response (Fig. 2a). The triple response in *Arabidopsis* seedlings is characterized by a shortened and thickened hypocotyl, an inhibition of root elongation, and the formation of an exaggerated apical hook (Guzman and Ecker, 1990). The readily distinguishable phenotype and the ability to screen thousands of seedlings on a Petri dish have greatly facilitated the identification of mutants that affect ethylene signalling in *Arabidopsis*. Mutations isolated using a screen for an altered triple response to ethylene fall into two main classes: (1) etiolated seedlings with minor or no phenotypic response upon ethylene application are termed *ethylene-insensitive* (*ein*) or *ethylene-resistant* (*etr*) mutants (2) mutants that display a constitutive triple response in the absence of ethylene (Kieber et al., 1993; Roman and Ecker, 1995) (Fig. 2b). The second class can be divided in two subgroups based on whether or not the triple response can be suppressed by inhibitors of ethylene perception and biosynthesis (such as silver thiosulfate and AVG). Mutants that are unaffected by these inhibitors are the constitutive triple response (*ctr*) mutants. On the contrary, mutants whose phenotype reverts to normal morphology are the ethylene-overproducer (*eto*) mutants, which are defective in the regulation of ethylene biosynthesis. The standard triple response screen is likely saturated

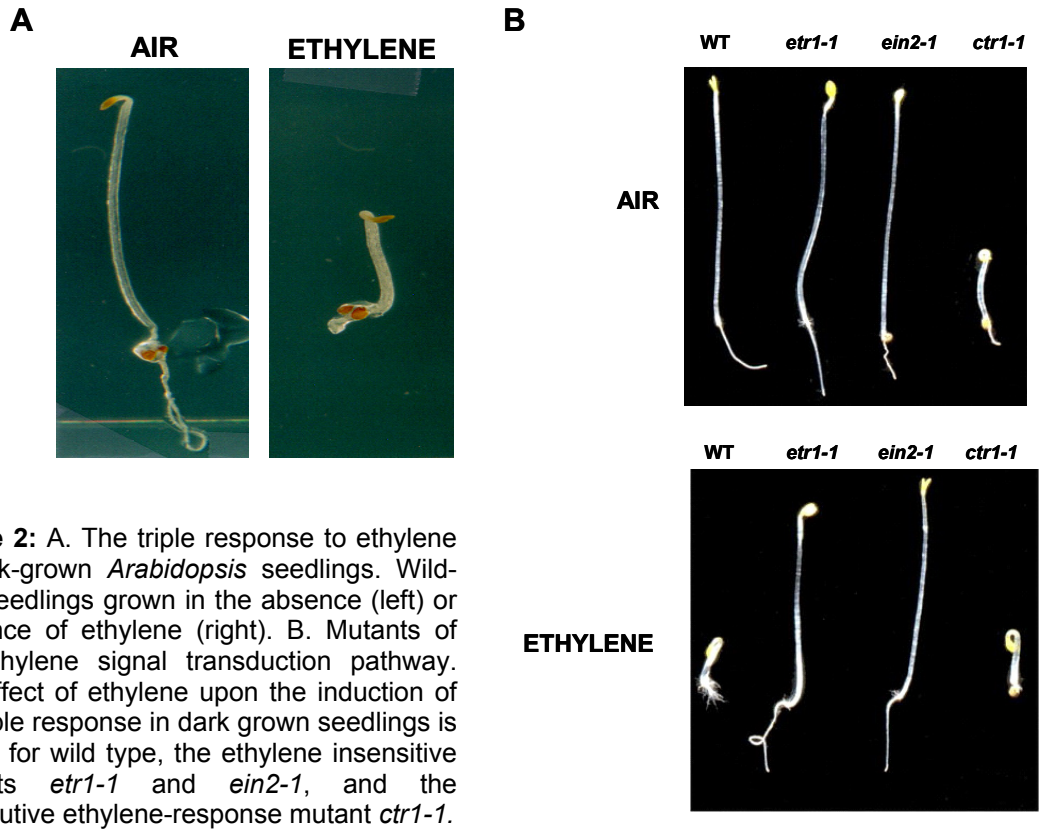


Figure 2: A. The triple response to ethylene of dark-grown *Arabidopsis* seedlings. Wild-type seedlings grown in the absence (left) or presence of ethylene (right). B. Mutants of the ethylene signal transduction pathway. The effect of ethylene upon the induction of the triple response in dark grown seedlings is shown for wild type, the ethylene insensitive mutants *etr1-1* and *ein2-1*, and the constitutive ethylene-response mutant *ctr1-1.*

for the identification of viable mutants that affect ethylene responses. However, refinements of the screen continue to yield results. One refinement is to screen for mutations that display an enhanced ethylene response at a low ethylene concentration. Using this screen, the enhanced-ethylene-response (*eer1*) mutant was isolated (Larsen and Chang, 2001). Recently, five components of the ethylene-response pathway (*wei1-wei5*) have been identified by using a low-dose screen for weak ethylene-insensitive mutants (Alonso et al., 2003a). Additionally, screening methods different from those using the triple response have been applied. One method uses the ethylene responsiveness to an antagonist of ethylene (a compound that interacts with the receptor but normally acts as an inhibitor of ethylene responses). The *responsive to antagonist* (*ran*) mutant was isolated using trans-cyclo-octene (TCO) as an antagonist (Hirayama et al., 1999). Moreover, light-grown *Arabidopsis* seedlings display an elongated hypocotyl in the presence of ethylene on a low nutrient medium (LNM), a response that is absent in the *etr* mutant and that is constitutively present in the *ctr* mutant in the absence of ethylene (Smalle et al., 1997) (Fig. 3). Using this response, the *alh* (*ACC-related long hypocotyl*) (Vandenbussche et al., 2003), *slo* (*slow*) (Zhang et al., in preparation) and the *eer2* mutant (De Paepe et al., in preparation; chapter 8 in this work) are isolated in our lab.

The gene products for many of the mutations affecting ethylene signal transduction have been identified (Fig. 4). The Raf-like kinase CTR1 represents the first step in a MAP

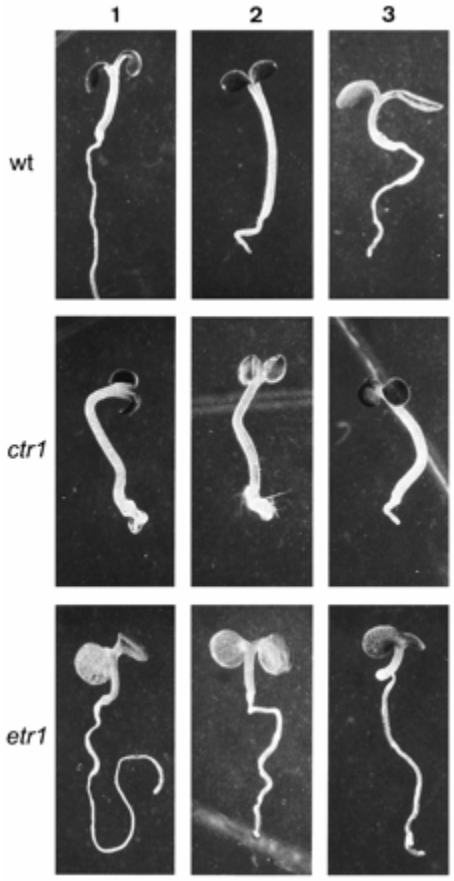


Figure 3: Effect of ACC treatment on seedlings of Col-0, *etr1-3*, and *ctr1-1* grown on LNM in the light. Column 1, LNM; column 2, LNM with 50 μM ACC; column 3, LNM with 50μM ACC and 100μM $AgNO_3$ (inhibitor of ethylene action). *(From Smalle et al., 1997)*

kinase pathway, a signalling cascade found in eukaryotic but not in prokaryotic systems (Kieber et al., 1993). The EIN3/EIL and ERF families of transcription factors are unique to plants (Ohme-Takagi and Shinshi, 1995; Chao et al., 1997). Several components of the ethylene signal transduction pathway contain transmembrane domains, these are the ethylene receptors (Schaller and Bleecker, 1995), the RAN1 protein (Hirayama et al., 1999), and EIN2 (Alonso et al., 1999). Although from the sequences alone it is not possible to determine to which membranes these components are localized, recent experimental evidence demonstrated that ETR1, one of the ethylene receptors, is localized to the endoplasmic reticulum (Chen et al., 2002). A genetically defined pathway for ethylene signal transduction has been determined by double-mutant analysis. Therefore, crosses between *ctr1* and the ethylene-insensitive mutants were analyzed to determine the order of gene action. Genetic epistasis studies of *Arabidopsis* signalling mutants revealed that *ETR1*, *ETR2* and *EIN4*, along with their homologues *ERS1* and *ERS2*, work upstream of *CTR1*, whereas *EIN2*, *EIN3*, AIN1/*EIN5*, and *EIN6* work downstream (Roman et al., 1995; Hall et al., 2000).

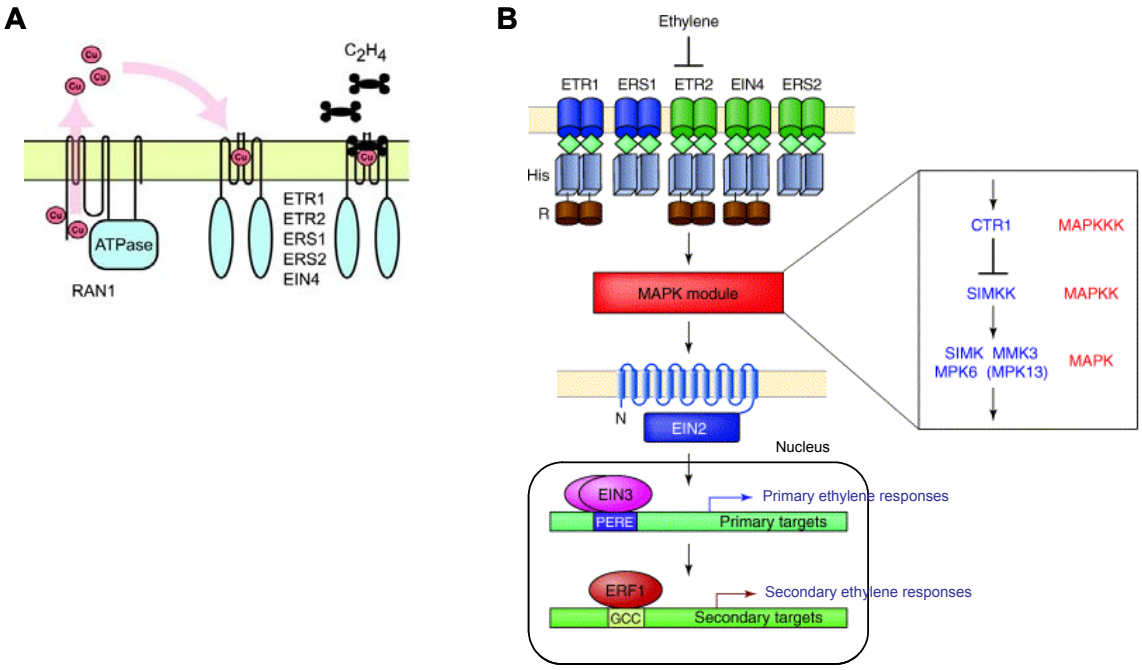
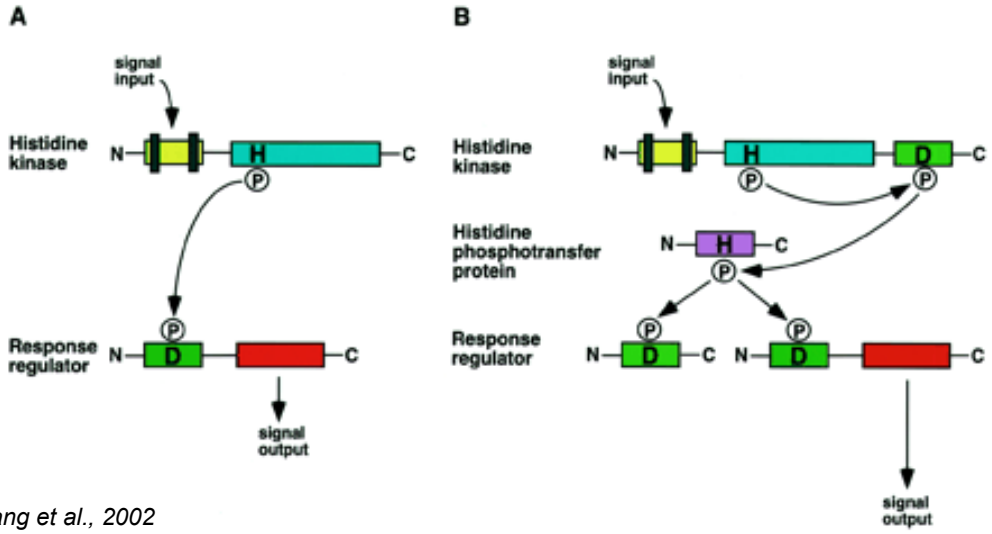


Figure 4: Model for ethylene signal transduction. A. Copper loading of ethylene receptors by RAN1. Copper is delivered to the ethylene receptor apoproteins. Upon coordinating copper, the ethylene receptors are competent for ethylene binding (*Modified picture from Schaller and Kieber, 2002).* B. The five ethylene receptors (ETR1, ERS1, ETR2, EIN4, ERS2) are thought to be dimers and are members of the two-component receptor family, which is characterized by a histidine kinase domain (His) and a receiver domain (R). The receptors are negative regulators of ethylene responses such that ethylene binding represses receptor signaling. CTR1, the next component downstream of the receptors is a Raf-like protein kinase and a negative regulator of ethylene responses. The findings of Ouaked et al., (2003) suggest that CTR1 might act in the MAPK module as presented in the picture. In the absence of ethylene, the CTR1 Raf-like kinase is activated, negatively regulating SIMKK (a MAPKK from *Medicago*). When CTR1 is inactivated by ethylene, SIMKK becomes activated and in turn activates two *Medicago* MAPKs (SIMK and MMK3) or the presumed *Arabidopsis* orthologs of SIMK and MMK3 (MPK6 and MPK13, respectively). The direct downstream targets of the MAPKs have yet to be determined. Inactivation of CTR1 results tin activation of EIN2, a positive regulator of ethylene responses, whose signaling mechanism is unknown. The EIN2 N-terminal transmembrane domain has similarity to the N-ramp family of metal ion transporters, and the hydrophilic C-terminus is novel. In the nucleus, an ethylene-dependent transcriptional cascade occurs. When activated by ethylene, members of the EIN3 transcription factor family bind as dimers to the primary ethylene response element (PERE) in the promoters of primary response genes such as *ETHYLENE-RESPONSE-FACTOR1* (*ERF1*). *ERF1* encodes an ethylene-response-element-binding-protein (EREBP). ERF1 and perhaps other EREBPs bind to the GCC-box of secondary response targets, such as *basic chitinase* and the defensin *PDF1.2*, activating their transcription (*Modified picture of Chang (2003)*).

1.2.1 Ethylene perception: the ethylene receptors ETR1, ETR2, ERS1, ERS2, and EIN4

Ethylene is perceived by a family of five membrane-localized receptors that are homologous to bacterial two-component histidine kinases. Sensor kinases and response regulators are the two principal players of the two-component signalling cascade (Stock et

al., 2000). The sensor kinase (receptor-like) component typically comprises two domains: an amino-terminal input domain, which perceives the signal, and a carboxyl-terminal histidine protein kinase (HPK) domain, which transmits the signal. The prototypical response regulator consists of a conserved receiver module and a variable output domain that mediates downstream responses. In bacteria the response regulator is usually transcription factor containing a conserved Asp-residue. Two-component pathways share the following His-Asp signalling mechanism: signal perception regulates autophosphorylation of a conserved His residue in the sensor kinase; the phosphoryl group is subsequently transferred to a conserved Asp residue in the receiver domain of the response regulator, thereby modulating the activity of the output domain (Fig.5A) (Hwang et al., 2002). In some cases, as in the ethylene receptors, the response regulator is not an independent polypeptide but a carboxyl-terminal domain of the sensor, in which case the receptor is called a hybrid HPK. Some hybrid HPKs transmit the signal via a multistep phosphorelay involving a third component known as a histidine-containing phosphotransfer (Hpt) domain protein. The Hpt protein serves as a phosphohistidine intermediate in the transfer of the phosphoryl group from a receiver domain to another receiver, resulting in a His-Asp-His-Asp phosphorelay (Fig. 5B).

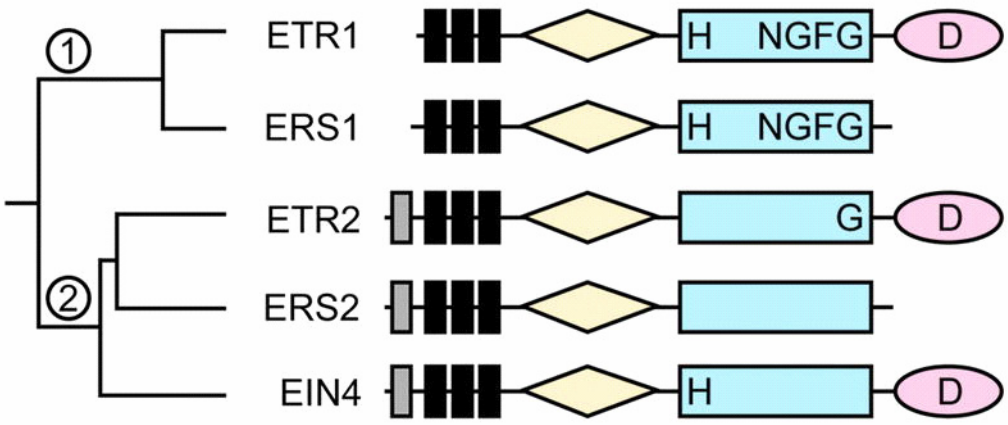


From Hwang et al., 2002

Figure 5: Schematic representation of the two-component and the multistep phosphorelay signalling systems. A. The prototypical two-component pathway uses a single phosphoryl transfer event between a His protein kinase and its cognate response regulator. B. The multistep His-to-Asp phosphorelay system in which a His-containing phosphotransfer protein serves as a phosphoryl acceptor and donor between the hybrid protein kinase and the response regulator. H, His; D, Asp; P, phosphoryl group

The ethylene receptor family has sequence similarity to both the sensor kinase and hybrid HPK of the two-component system. In *Arabidopsis*, all five ethylene receptors (ETR1, ERS1, ETR2, EIN4, ERS2) have an amino-terminal ethylene-binding domain, and the

carboxyl-terminal portion has similarity to HPKs. Three of the receptors (ETR1, ETR2, and EIN4) also have a covalently attached carboxyl-terminal receiver domain, and are therefore considered to be hybrid HPKs. ERS1 and ERS2 lack a receiver domain, suggesting that these receptors may have a distinct signalling circuit, or perhaps they signal to a receiver domain of one of the hybrid HPK receptors. There are enough differences to support the existence of two subfamilies of ethylene receptors (Fig. 6) (Hua et al., 1998). Subfamily 1 is composed of ETR1 and ERS1. Subfamily 2 is composed of ETR2, ERS2, and EIN4. In subfamily 1 there is complete conservation of the residues considered essential for histidine kinase activity. On the other hand in subfamily 2 the histidine kinase domain lacks some essential residues. This raises the question of whether the latter possess histidine kinase activity. The expected target of two-component signalling, namely an output domain regulated by a receiver domain is unknown. Interestingly, the *ers1etr1* double loss-of-function mutant (subfamily I receptors mutant) could not be compensated by overexpression of any of the three subfamily II receptors, whereas subfamily I constructs restored normal growth, supporting the idea that subfamily I receptors play a unique and necessary role in ethylene signalling (Wang et al., 2003).



From Schaller and Kieber, 2002

Figure 6: The Ethylene Receptor Family. Primary structures of the five-member family are indicated. Black bars represent transmembrane segments. Gray bars represent putative signal sequences. Diamonds indicate GAF domains. Rectangles indicate histidine kinase domains. Ovals indicate receiver domains. The conserved phosphorylation sites upon histidine (H) and aspartate (D) are indicated if present. Conserved motifs (NGFG) within the histidine kinase domain are indicated if present. There are two subfamilies of ethylene receptors (subfamily 1 and 2) based on sequence and phylogenetic analysis.

However, transformation of either the *ers1-2 etr1-6* or *ers1-2 etr1-7* mutant with a kinase-inactivated ETR1 genomic clone also resulted in complete restoration of normal growth and ethylene responsiveness in the double-mutant background, leading to the conclusion that canonical histidine kinase activity of receptors is not required for ethylene receptor

signalling. Different possibilities for ethylene receptor signalling remain. A first possibility can be that a novel autophosphorylation mechanism occurs in subfamily I and II receptors. Secondly, a novel signalling mechanism that does not require phosphotransfer may have evolved in the ethylene receptor family (Wang et al., 2003). It is suggested that the Raf-related CTR1 could fulfil this role. In this case, the physical interaction of ETR1 and ERS1 transmitter domains with the regulatory domain of CTR1 could provide the direct propagation of conformational change from receptors to the CTR1 catalytic domain. Alternatively, conformational changes in receptors could recruit and/or release CTR1 from a receptor complex, analogous to the recruitment of Raf kinases into receptor complexes in animals (as explained in chapter 2.2.3). Further signalling by CTR1 could occur through autophosphorylation, transphosphorylation of receptors, or phosphorylation of downstream effectors (Wang et al., 2003). A possible reason for the retention of the histidine kinase activity in subfamily I receptors may be to provide fine-tuning of the signalling pathway rather than functioning as the primary mechanism for signal transduction.

1.2.2 Ethylene binding to the receptors

The ethylene receptor ETR1 is the founding member of the ethylene-receptor family and has been characterized in the most detail. ETR1 is a membrane protein of 738 amino acid residues and contains three aminoterminal transmembrane domains that encompass the ethylene-binding site (Schaller et al., 1995). A sequence of unknown function, which has similarity to GAF domains, is situated between the ethylene-binding site and the HPK domain. In some proteins, the GAF domain is involved in binding of cGMP (Aravind and Ponting, 1997). The functional unit for ethylene perception is likely to be a receptor dimer (Schaller et al., 1995). In the case of ETR1, homo-dimerization is mediated in part by cysteine residues near the amino terminus that are capable of forming disulfide bonds (Schaller et al., 1995). All five ethylene receptors contain these cysteine residues. Since homodimerization of ETR1 and ERS1 has been observed in plants (Schaller et al., 1995; Hall et al., 2000), receptors that do not have the receiver domain, ERS1 and ERS2, have been postulated to use the receiver domains of other proteins by forming heterodimers with them (Hua et al, 1998). Ethylene binding has been demonstrated for both ETR1 and ERS1 by using a yeast cell expression system (Schaller and Bleecker, 1995; Hall et al., 2000). The binding occurs with a dissociation constant of 0.04 μl/L gaseous ethylene and a half-life of 12 hours, both of which are consistent with rates observed in ethylene-binding/response assays in plants (Chen and Bleecker, 1995). The binding site appears to consist of three hydrophobic regions, which possibly serve as a membrane anchor

(Schaller and Bleecker, 1995; Rodriguez et al., 1999). ETR1 was shown to bind ethylene only in the presence of copper ions that are coordinated by two conserved amino acids (Cys65 and His69) (Schaller and Bleecker, 1995; Rodriguez et al., 1999). Further evidence for a role of copper in ethylene signaling comes from the characterization of the *RAN1 (responsive to antagonist)* gene (Hirayama et al., 1999). Two mutant alleles, *ran1-1* and *ran1-2*, were identified in a screen for mutants that displayed an ethylene-like triple response upon treatment with the ethylene antagonist trans-cyclo-octene. In contrast to the *ran1-1* and *ran1-2* alleles that are morphologically indistinguishable from wild-type plants, the *ran1-3* allele results in a rosette-lethal phenotype (Hirayama et al., 1999; Woeste and Kieber, 2000). The lethal phenotype is thought to be caused either by general effects due to the reduced copper availability for other copper-utilizing enzymes, or by disruption of ethylene receptor function. *RAN1* codes for a copper transporting P-type ATP-ase, such as the yeast Ccc2p and human Menkes/Wilson disease proteins. The RAN1 protein is located in intracellular membrane compartments and is believed to deliver copper ions from storage sources inside the cells. Ethylene receptors were originally identified based on mutations that resulted in a dominant ethylene-insensitive phenotype (Chang et al., 1993; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998). These mutations turned out to be missense mutations within the sensory domain of the receptors. As for the *etr1-1* mutant, an amino acid crucial for ethylene binding (conversion of Cys65 to Tyr) is affected. Therefore, the *etr1-1* mutant is unable to chelate copper, and as a consequence can not bind ethylene (Rodriguez et al., 1999). Also *etr1-3* and *etr1-4* have been found to reduce the ethylene binding (Hall et al., 1999). The *etr1-2* mutant, which displays a missense mutation in the third transmembrane domain, does not eliminate ethylene binding and thus appears to impede intramolecular transduction of the ethylene signal (Hall et al., 1999).

An interesting feature of the ethylene receptors is that they are negative regulators of the ethylene-response pathway. The negative regulation by the receptors was revealed through the analysis of loss-of-function mutants in four of the five members of the family (Hua and Meyerowitz, 1998). Loss-of-function mutants for *ETR1*, *ETR2*, and *EIN4* were identified among revertants of dominant ethylene-insensitive mutants for these genes, and a loss-of-function *ERS2* allele was obtained by a T-DNA insertion. Single ethylene receptor mutants showed wild-type phenotypes, indicating that these receptors have redundant functions. However, when receptor mutants were crossed, the resulting homozygous triple and quadruple loss-of-function mutants displayed constitutive ethylene responses. Since the absence of receptors resulted in constitutive responses, the wild-

type receptors must be negative regulators of ethylene responses. This suggests a model in which the receptors repress ethylene responses when no ethylene is bound. Consequently, when ethylene is bound, the receptors are inactivated and the pathway is derepressed, leading to ethylene responses. One explanation for the basis of the dominance of the ethylene-insensitive receptor mutations is that the mutant gain-of-function receptors are somehow hyperactive (Chang and Stadler, 2001). This hyperactive signaling by the dominant mutant receptors might be achieved via higher order complexes of ethylene receptors, such that the locked signalling conformation of a mutant receptor dictates the conformation of the associated receptors. Conceivably, a dominant mutant ethylene receptor might catalyze transphosphorylation of wild type ethylene receptors, which would otherwise be inactive (Chang and Stadler, 2001).

Since null mutants of the receptor genes appear to be wild type and the expression patterns of the receptor genes overlap widely, one can argue why there are multiple ethylene receptors. Families of ethylene receptor genes are found not only in *Arabidopsis*, but in other plants as well. Previously Hua *et al.* (1998) proposed that the different ethylene receptors may possess different ethylene binding affinities and signaling activities, enabling the plant to respond to a broad spectrum of ethylene concentrations. In addition, the ethylene receptors are differentially regulated by ethylene and perhaps also by other environmental or developmental factors. Ethylene itself has been found to regulate transcription of the receptors *ERS1*, *ERS2*, and *ETR2*. The differential regulation of expression of the receptor gene family may provide a mechanism to achieve differential sensitivities even in the same response under different conditions (Hua et al., 1998). The upregulation of receptors in response to ethylene might provide a mechanism for adaptation to ethylene; since the half-life of ethylene binding is very long (12.5 hours), an increase in the number of unbound receptors could result in desensitization of the pathway and may enable the plant to react to changes in ethylene concentration.

1.2.3 Linking the ethylene receptors to CTR1, a MAPKKK

The first known component downstream of the ethylene receptors is CTR1. The *CTR1* gene encodes a protein of 821 amino acids (Kieber et al., 1993). The recessive nature and constitutive phenotype of the *ctr1-1* mutant indicate that CTR1 is a negative regulator of downstream signalling events (Kieber et al., 1993). Cloning of the *CTR1* gene revealed that it belongs to the Raf family of Ser/Thr protein kinases that initiate mitogen-activated protein (MAP)-kinase signaling cascades in mammals (Kieber et al., 1993). Based on this similarity, it was suggested that CTR1 may function as a mitogen-activated protein kinase

kinase kinase (MAPKKK). In animals, MAPKKKs are typically regulated by tyrosine-kinase receptors or by G-protein-coupled receptors (Blumer and Johnson, 1994). Upon ligand binding, the GTP-bound small G protein Ras recruits Raf to the plasma membrane and initiates the MAPK phosphorylation cascade. CTR1 lacks the corresponding domain for Ras binding in Raf, suggesting a different mode of action in plants. There are two known examples of pathways that combine a two-component system with a MAPK pathway. One is the *S. cerevisiae* osmosensing pathway and the other is the *S. pombe* stress-response pathway (Posas et al., 1996; Shieh et al., 1997; Buck et al., 2001). Thus far, no intermediate components have been identified genetically or biochemically to act between the receptors and the CTR1 kinase. In fact, yeast two-hybrid and in vitro binding have shown that both the kinase domain and receiver domain of ETR1 and the kinase domain of ERS1 (which lacks a receiver domain) can directly interact with CTR1 (Clark et al., 1998). Physical interactions of the subfamily II ETR2 transmitter domain were also demonstrated, but are much weaker than those reported for ETR1 and ERS1 (Cancel and Larsen, 2002). Moreover, interaction between ethylene receptors and CTR1 has recently been shown in plants (Gao et al., 2003). It is demonstrated that native CTR1 is localized to the endoplasmic reticulum of *Arabidopsis* and that this localization arises due to interactions with ethylene receptors. The function of the N-terminal part of CTR1, which encodes a novel protein domain, was investigated by Huang et al. (2003) (Huang et al., 2003). Deletion of the N-terminal domain did not elevate the kinase activity of CTR1, indicating that this domain does not autoinhibit kinase function in vitro. One missense mutation, *ctr1-8* was found to result from an amino acid substitution within a conserved motif in the N-terminal domain. In vitro, *ctr1-8* has no detectable effect on the kinase activity of CTR1, but rather disrupts the interaction with the ethylene receptor ETR1, suggesting that CTR1 interacts with ETR1 in vivo and that this association is required to turn off the ethylene signalling pathway.

All together, these results raise several possibilities for regulation of CTR1 activity (Schaller and Kieber, 2002). A first possibility is that CTR1 could directly respond to the phosphorylation state of the receptors. Secondly, CTR1 could be regulated by conformational changes in the receptors. It is proposed that dimerization of the ETR1 receiver domain occurs in the unphosphorylated state, and that phosphorylation would cause monomerization (Muller-Dieckmann et al., 1999). The monomerization of the ETR1 receiver domain upon ethylene-induced phosphorylation might inactivate CTR1 (Chang and Stadler, 2001). A third way to regulate CTR1 activity might be through intermediary proteins when CTR1 is complexed with the receptors. Gao et al. (2003) demonstrated that

CTR1 is part of a signalling complex with the ethylene receptor ETR1 in plants. Single receptor mutants did not result in significant loss of CTR1 from the membrane, double and triple mutant combinations did, thereby implicating multiple receptors in the membrane localization of CTR1.

Recently, the work of Ouaked et al. showed evidence that a MAPK cascade is part of ethylene signalling (Ouaked et al., 2003). They demonstrated that two MAPKs are activated by ACC in *Medicago* and *Arabidopsis*. In *Medicago* SIMK and MMK3 showed strong activation in ACC-treated cells, whereas in *Arabidopsis* this was the case for MPK6 and a 44kDa protein, probably corresponding to MPK13. Furthermore, their analysis showed that the MAPKK SIMKK specifically mediates ACC-induced activation in *Medicago*. Transgenic *Arabidopsis* plants with hyperactive SIMKK showed constitutive MAPK activation, enhanced ethylene-induced gene expression and a triple response phenotype in the absence of ACC. All together, these results indicated that a MAPK pathway is part of ethylene signalling in plants.

Several observations indicate that the ethylene signal transduction pathway is not completely dependent upon activity of CTR1. First, the *ctr1* null mutants are still capable of responding to ethylene (Larsen and Chang, 2001). Second, plants containing loss-of-function mutations in four ethylene receptors display a more severe phenotype than *ctr1* loss-of-function mutations (Hua and Meyerowitz, 1998). Possible explanations for these results are that other CTR1-like proteins act in the ethylene signal transduction pathway or that also a “traditional” two-component signalling pathway exists.

1.2.4. EIN2, an NRAMP-like protein

Genetic epistatic analysis of ethylene response mutants has shown that EIN2 acts downstream of CTR1. Null mutants in *EIN2* result in the complete loss of ethylene responsiveness throughout plant development, suggesting that EIN2 is an essential positive regulator in the ethylene signalling pathway. EIN2 encodes an integral membrane protein of 1294 amino acids (Alonso et al., 1999). EIN2 contains 12 predicted transmembrane domains in the aminoterminal part of the polypeptide. This region exhibits significant similarity to the Nramp family of cation transporters such as the yeast Smf1p, and mammalian DCT1 proteins. The C-terminal hydrophilic part has no homology to any known protein, although it does contain motifs typically involved in protein-protein interactions. Overexpression of the carboxyterminal domain of the protein (EIN2 CEND) in an EIN2 null background results in constitutive activation of some but not all ethylene responses. The transformed plants displayed constitutive ethylene-response phenotypes

as adults and constitutively expressed ethylene-regulated genes, but expression of the EIN2 carboxyterminal region was unable to induce the triple response in dark-grown seedlings. Furthermore, the transformed plants were restored in their ability to respond to paraquat and JA, but not to ethylene. Based on these results, it was hypothesized that the aminoterminal end of EIN2 represents an input domain, interacting with upstream signalling factors, while the carboxyterminal region is required for transducing the signal to the downstream components. The *ein2* mutants exhibit the strongest ethylene-insensitive phenotype of all ethylene insensitive mutants isolated in *Arabidopsis*. This indicates that EIN2 plays a critical role in ethylene signalling. However, it is still not clear how EIN2 functions in ethylene signalling. There is no precedent for an Nramp-like protein operating downstream of a MAP kinase pathway. Moreover, it is still questionable whether EIN2 functions in an analogous manner. Previous experiments to detect metal transporting activity in EIN2 have failed, and where other members of the *Arabidopsis* Nramp-like family are able to complement metal-uptake deficient yeast strains, this is not the case for EIN2 (Thomine et al., 2000). Interestingly, *ein2* mutants have been independently isolated in several different genetic screens designed to identify components of other signalling pathways. For example, *ein2* mutants have been found in screens for defects in auxin transport inhibitor resistance (Fujita and Syono, 1996), cytokinin response (Su and Howell, 1992), ABA hypersensitivity (Beaudoin et al., 2000; Ghassemian et al., 2000) and delayed senescence (Oh et al., 1997). In addition, *ein2* mutants show altered sensitivity to several bacteria and fungal pathogens. Hence, EIN2 has been proposed to lie at the crossroad of multiple hormones and stress response pathways.

1.2.5. Nuclear events

Many ethylene responses involve changes in gene expression. Evidence for nuclear regulation in the ethylene signal transduction was given by the cloning of *EIN3* (Chao et al., 1997). EIN3 encodes a nuclear-localized protein of 628 amino acids and contains acidic, proline-rich, and glutamine-rich domains which have been found in transcriptional activation domains. *EIN3* belongs to a multigene family with six members in *Arabidopsis*. Besides EIN3, also EIN3-like 1 (EIL1) and EIL2 can rescue the *ein3* mutant phenotypes, which indicates that not only EIN3 but also EIL1 and EIL2 are involved in ethylene signalling. This also explains why null mutations in *ein3* cause only partial ethylene insensitivity. The isolation of *wei5/eil1* confirmed that *EIL1* is a component of ethylene signalling cascade (Alonso et al., 2003a). Interestingly, *eil1 ein3* double mutant seedlings were almost completely ethylene-insensitive and indistinguishable from the ethylene-

response null mutant *ein2-5*, indicating that *EIL2-EIL5* genes may not contribute to the ethylene response at this stage of development. Overexpression of *EIN3* in an *ein2* null background caused a constitutive ethylene response phenotype, similar to the overexpression of the EIN2 CEND, confirming that EIN3 acts downstream of EIN2. Since EIN3 expression is not induced by ethylene, EIN3 may be regulated by ethylene at the protein level. Evidence for the latter is recently described (Guo and Ecker, 2003; Potuschak et al., 2003). In the absence of ethylene, EIN3 is quickly degraded through a ubiquitin/ proteasome pathway mediated by two F-box proteins, EBF1 and EBF2 (for EIN3-binding F box protein 1 and 2). EBF1 overexpression resulted in plants insensitive to ethylene. In contrast, *ebf1ebf2* double mutants showed constitutive ethylene responses. Altogether, these studies revealed that a ubiquitin/proteasome pathway negatively regulates ethylene responses by targeting EIN3 for degradation. EIN3 degradation needs to be switched off to allow EIN3 accumulation after ethylene stimulation. Several mechanisms can be envisaged to explain how ethylene regulates EIN3 stability. Either the $SCF^{EBF1/EBF2}$ complexes are negatively regulated after ethylene perception to allow EIN3 accumulation. Or, EIN3 is directly protected from the $SCF^{EBF1/EBF2}$ ubiquitin protein ligases ((Potuschak et al., 2003). In addition, another study demonstrated that glucose accelerated the degradation of EIN3 (Yanagisawa et al., 2003). The antagonistic relationship between ethylene and glucose was identified before by the genetic and phenotypic analyses of *Arabidopsis* mutants with *glucose-insensitive* (*gin*) and *glucose-oversensitive* (*glo*) phenotypes (Zhou et al., 1998). The ethylene-insensitive *etr1* and *ein2* mutants displayed *glo* phenotypes, while ctr1 was allelic to *gin4* (Leon and Sheen, 2003).

To date, the ethylene-inducible gene *ERF1* is the only known direct target of EIN3. EIN3 dimers interact with a unique palindromic repeat element in the promoter of ERF1, which is termed the primary ethylene response element (PERE). Homodimers of EIL1 an EIL2 are also capable of binding to this DNA sequence in vitro, but no heterodimerization between these proteins and EIN3 has been reported (Solano et al., 1998). *ERF1* belongs to a large family of plant-specific transcription factors referred to as ethylene response element binding factor (ERF) proteins (Fujimoto et al., 2000), a family that has also been referred to in the literature as ethylene response element binding proteins (EREBPs) (Ohme-Takagi and Shinshi, 1995), which are capable of binding to a secondary ethylene response element, the GCC-box. This sequence was determined to be essential for the expression of several *PR* (pathogenesis-related) genes (Zhou et al., 1998; Fujimoto et al., 2000). Some *ERF* genes encode transcriptional activators, while others encode transcriptional repressors. The *AtERF* genes are differentially regulated by ethylene and by abiotic stress

conditions, such as wounding, cold, high salinity, or drought, via EIN2-dependent or -independent pathways (Fig. 7) (Fujimoto et al., 2000). Interestingly, some ERFs contain GCC boxes in their promoter, indicating that these ERFs could be targets for other members of the ERF family (Solano et al., 1998). The uncovering of this transcriptional cascade represents an important step in unravelling the different players by which ethylene regulates gene expression. Furthermore, this transcriptional cascade with multi-member families allows modulation by other regulatory pathways at many points. However, a number of ethylene-response genes possess neither the PERE nor the GCC-box in their promoters. This observation indicates that transcription factors unrelated to either EIN3 or ERF1 may also be involved in the transcriptional control of ethylene signalling.

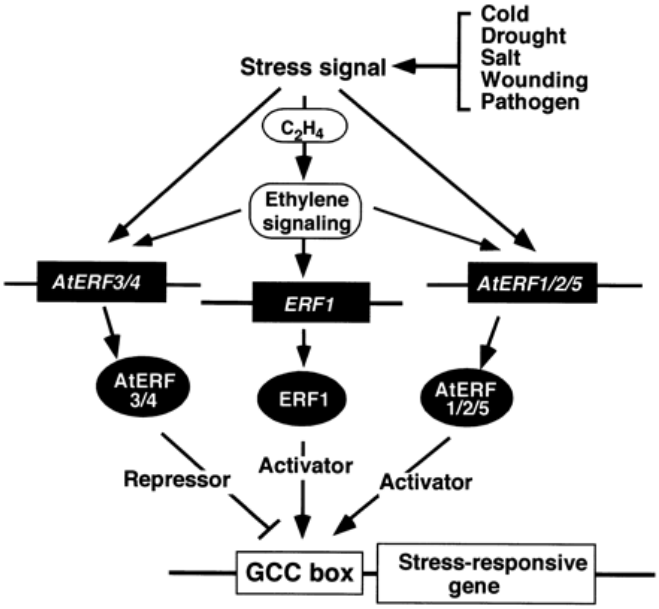


Figure 7: A Model for GCC Box–Mediated Stress Signal—Dependent Transcription by ERF Proteins in *Arabidopsis*. After reception of a stress signal by plants, *ERF* genes may be upregulated via an ethylene-dependent pathway or an ethylene-independent pathway. *ERF* genes such as *ERF1* are induced by ethylene and can interact with GCC box–containing stress response genes. AtERF1, AtERF2, or AtERF5 may activate a specific subset of GCC box–containing genes, whereas AtERF3 and AtERF4 may repress the expression of these genes. These data suggest that the ERF proteins may act as factors responsive to extracellular signals and that they are involved in regulating a subset of GCC box–containing stress response genes

From Fujimoto et al., 2000

1.2.6 Other genetically defined components in ethylene signalling

Besides the well characterized mutants described in the previous sections, other mutations have also been identified that affect ethylene signalling. For some of these mutants it is not yet clear where they fit into the signal transduction pathway, while for other mutants only a subset of the ethylene responses are affected, indicating that they function downstream of the primary signal transduction pathway.

Based on double-mutant analysis the ethylene-insensitive mutants *ain1/ein5*, *ein6* and *ein7* (Van der Straeten et al., 1992; Roman et al., 1995) function downstream of CTR1. *ain1/ein5* and ein6 are recessive; *ein7* is semidominant. Phenotypically, these mutants display less severe ethylene insensitive phenotypes as observed for the *ein2-1* mutation.

In the recessive mutants *aux1* and *eir1* the hypocotyl is responsive to ethylene, but the roots show partial ethylene-insensitivity and display an altered gravitropic response (Roman et al., 1995). *aux1* plants are resistant to auxin, but *eir1* plants are responsive to auxin. AUX1 is identified as an auxin influx carrier functioning in the transport and redistribution of auxin in the plant (Bennett et al., 1996; Marchant et al., 1999). Ethylene insensitivity in the roots of *aux1* and *eir1* may relate to the role of ethylene in regulating auxin transport (Suttle, 1988; Roman and Ecker, 1995). Another mutant, *hls1*, lost the ability to form a pronounced apical hook in response to ethylene in the dark (Roman and Ecker, 1995; Lehman et al., 1996). The *hookless* morphology could be phenocopied by adding auxin or auxin-transport inhibitors (Lehman et al., 1996). Thus, again this suggests a link between auxin and ethylene signaling. *HLS1* encodes a putative N-acetyltransferase and could potentially acetylate a compound involved in auxin signalling or transport (Lehman et al., 1996). In addition, the *alh1* (ACC-related long hypocotyl 1) is also altered in the cross talk between ethylene and auxins. The *alh1* mutant displayed a longer hypocotyl in the light than the wild type in the absence of ethylene; this feature could be reverted by auxin transport inhibitors (Vandenbussche et al., 2003).

Several novel ethylene-related mutants have recently been identified. Interestingly, these mutants were not identified by using the triple response assay, but by novel genetic screens. By using subtreshold levels of ethylene the *eer1* (*enhanced ethylene response*) mutant was identified (Larsen and Chang, 2001). The *eer1* mutant displays increased ethylene sensitivity in the hypocotyl and stem but reduced sensitivity in the root. Molecular cloning of *eer1* revealed that its mutant phenotype results from a loss-of-function mutation in RCN1, one of the three PP2A A regulatory subunits in *Arabidopsis* (Larsen and Cancel, 2003). A role for PP2A in the modulation of CTR1 activity is proposed.

Five ethylene-insensitive loci were identified by using a low-dose screen for weak ethylene insensitive mutants (wei1-wei5) (Alonso et al., 2003a). *wei1*, *wei2*, and *wei3* seedlings showed hormone sensitivity only in roots, whereas *wei4* and *wei5* displayed insensitivity in both roots and hypocotyls. The *wei1* mutant harbored a recessive mutation in *TIR1*, which encodes a component of the SCF protein ubiquitin ligase involved in the auxin response. During the past few years, it has become clear that one of the major processes regulating hormonal responses is protein degradation. Previously, a role for ubiquitin-mediated protein degradation has been demonstrated in auxin, jasmonate, cytokinin, absiscic acid and GA signalling (Kepinski and Leyser, 2002; Smalle et al., 2002; McGinnis et al., 2003; Smalle et al., 2003). As for ethylene signalling, very recently evidence was shown for EIN3-dependent regulation of ethylene signalling by two F box proteins : EBF1 and EBF2

(Guo and Ecker, 2003; Potuschak et al., 2003). Additional evidence that proteolytic degradation is part of ethylene signalling is demonstrated in this thesis (see Chapter 2 and 4).

wei4 resulted from a mutation in the ethylene receptor ERS1 and *wei5* was caused by a mutation in the *EIN3*-related transcription factor *EIL1*. Genetic mapping studies indicated that *wei2* and *wei3* correspond to previously unidentified component in the ethylene pathway.

Cloning and characterization of these unidentified genes will further expand our knowledge of the ethylene signal transduction pathway.

1.3 CROSS-TALK IN PLANT HORMONE SIGNALLING

As described in the previous sections, genetic screens have been very useful in identifying factors involved in ethylene signal transduction. However, although these screens were originally designed to identify specific components in ethylene signalling, mutations in these genes often confer changes in sensitivity to other hormones. Moreover, alleles of mutations in ethylene signalling have also been recovered in screens using auxin transport inhibitors, resistance to cytokinin application or in screens for suppressor and enhancer mutants of abscisic acid (ABA) mutants or to uncover regulators of sugar metabolism (Zhou et al., 1998; Vogel et al., 1998b; Beaudoin et al., 2000; Ghassemian et al., 2000). Together, these facts indicate that the linear representation of the hormone signalling pathways controlling a specific aspect of plant growth and development is not sufficient, and that hormones interact with each other and with a variety of developmental and metabolic signals. Modulation of hormone sensitivity can happen in different ways.

In some cases addition of one hormone can influence the biosynthesis of another. As described before, previous studies showed that cytokinin treatment increased the stability of ACS5, which is one of the members of the *Arabidopsis* gene family that encodes 1-aminocyclopropane-1-carboxylate synthase, the first enzyme in ethylene biosynthesis (Chae et al., 2003). As a consequence, many of the growth defects attributed to cytokinin are the result of ethylene overproduction. This also explains cytokinin insensitivity of *ein2* mutants, because mutants insensitive to ethylene obviously also are insensitive to exogenous cytokinin (Vogel et al., 1998a). In addition, auxin has been shown to stimulate ethylene biosynthesis at the level of transcription of genes encoding ACC synthase (Abel et al., 1995; Yamagami et al., 2003).

A second possibility of cross-talk resides in an integration of signal transduction routes. In this case the physiological response is the result of complex interactions between the

different signalling pathways. Auxin and ethylene co-ordinately regulate several developmental programs in plants. For example, in *Arabidopsis* auxin and ethylene have been described to regulate apical hook formation, root hair elongation, root growth and hypocotyl phototropism (Lehman et al., 1996; Pitts et al., 1998; Harper et al., 2000; Rahman et al., 2001). Nevertheless, it is often unclear whether developmental effects attributed to auxin are solely due to this hormone or rather mediated by ethylene or resulting from a synergistic interaction between both hormones. Moreover, cross-talk is readily apparent in the pathogen defence response in plants which is coordinated by ethylene, jasmonic acid (JA) and salicylic acid (SA). These three signalling factors are sometimes required individually and sometimes in concert for mobilizing defence responses to different pathogens (Glazebrook, 1999). Previous studies have shown that both ethylene and JA are required for the induction of the defensin gene *PDF1.2* in response to the avirulent fungal pathogen *Alternaria brassicicola* (Penninckx et al., 1998). In addition, a microarray analysis supported the coordination between these three signalling pathways because a big overlap in gene expression, especially between jasmonate and ethylene, was observed (Schenk et al., 2000).

Interactions between the hormone signalling pathways can also be dependent upon the developmental state of the plant and the specific response evaluated. Genetic analysis of ethylene and ABA interactions suggested that these hormones antagonize each other at the level of germination. Previously, two independent screens designed to discover mutants involved in ABA responsiveness identified ethylene signalling mutants (Beaudoin et al., 2000; Ghassemian et al., 2000). *era3* mutants, which were originally identified as ABA hypersensitive, were found to be allelic to *ein2.* Furthermore, *ctr1* and *ein2* mutants were identified as enhancers and suppressors of *abi1* mutants, respectively. Other ethylene insensitive mutants also showed increased ABA responsiveness leading to the conclusion that ethylene is a negative regulator of ABA signalling in *Arabidopsis* seeds. Ethylene appears to promote seed germination by altering endogenous ABA levels and/or by decreasing the sensitivity of the seeds to ABA. After germination, ABA and ethylene signalling display complex interactions. Mutations in the ethylene-insensitive mutant *etr1* reduced the sensitivity of roots to exogenous ABA, indicating that both hormones act additively with respect to root growth (Beaudoin et al., 2000; Ghassemian et al., 2000). Therefore, models in which they act in the same or parallel pathways are proposed. However, ethylene-overproducing mutants have decreased ABA sensitivity, implying another antagonistic interaction. One suggested explanation for this apparent inconsistency is that ABA inhibits root growth by signalling through the ethylene response

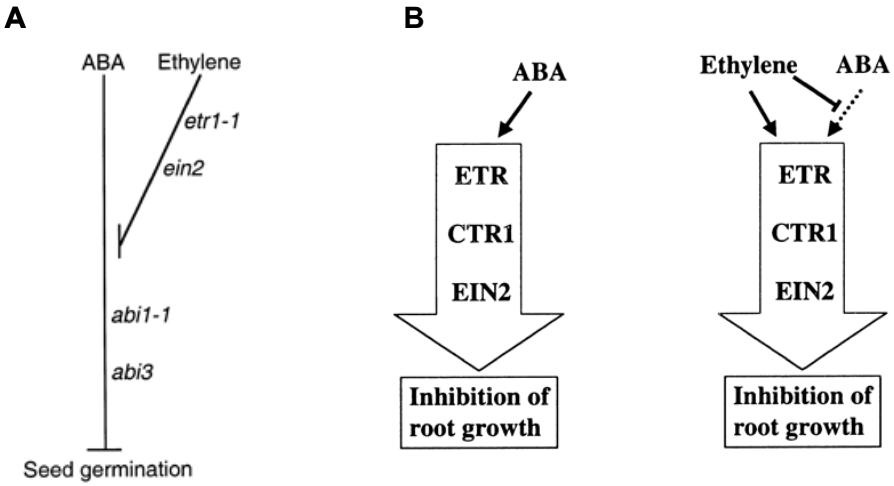


Figure 5: Hypothetical model for the interactions between the ABA and ethylene signalling cascades. A. Regulation of seed germination. Ethylene promotes seed germination by altering endogenous ABA levels and/or by decreasing sensitivity to endogenous ABA in imbibed seeds. The *abi1-1* and *abi3* mutants are ABA insensitive. (*Modified picture from Beaudoin et al., 2000*). B. Hypothetical model for the role of ABA and ethylene in regulating root growth in *Arabidopsis.* Left panel: in the absence of ethylene, one way for ABA to inhibit root growth is by signalling through the ETR1 response pathway. Therefore mutations in the ethylene pathway interfere with both ABA and ethylene signalling. Right panel: In the presence of ethylene, ABA is unable to use this pathway. Thus, mutations that increase ethylene synthesis confer an ABA-insensitive root phenotype (*Modified picture from Ghassemian et al., 2000*).

pathway, but is unable to use this pathway in the presence of ethylene (Ghassemian et al., 2000). Furthermore, both hormones do not appear to interact in ABA-regulated processes such as stomatal closure or induction of some ABA-response genes.

By investigating root growth and apical hook formation, regarded as two of the hallmarks of the traditionally ethylene triple response, a recent study demonstrated that ethylene regulates these phenomena at least in part via alteration of the properties of DELLA nuclear growth repressors (Achard et al., 2003; Vriezen et al., 2004). DELLA proteins were first described as gibberellin (GA) signalling components (Peng et al., 1997; Silverstone et al., 2001; Lee et al., 2002; Wen and Chang, 2002). Moreover, recent experiments have shown that auxin promotes root growth by modulating DELLA function (Fu and Harberd, 2003). Therefore it is suggested that DELLA proteins provide a connection between ethylene, auxin, and GA. Interaction between auxin, ethylene and gibberellin also was shown to occur in the promotion of hypocotyl growth and stomatal development in light-grown *Arabidopsis* seedlings (Saibo et al., 2003).

In the future, comparison of genetic interaction maps with patterns based on transcript profiling and other genomics technologies may allow a more comprehensive representation of hormone interactions within the cell.

1.4 ETHYLENE IN PLANT DISEASE RESISTANCE AND ABIOTIC STRESSES

Besides its physiological roles in different developmental stages, ethylene is also a stress hormone. Its synthesis is induced by a variety of stress signals, such as mechanical wounding, chemicals and metals, drought, extreme temperatures, and pathogen infection (Kende, 1993; Johnson and Ecker, 1998). However, depending on the type of pathogen and plant species and on the offensive strategies of the pathogen, the role of ethylene can be essentially different. Ethylene-insensitive signalling mutants may show either increased susceptibility or increased resistance. For example, in *Arabidopsis*, the *ein2-1* mutant developed only minimal disease symptoms when susceptibility was monitored with virulent *Pseudomonas syringae pv. tomato* or *Xanthomonas campestris pv. campestris*, whereas wild type plants were susceptible to these strains (Bent et al., 1992). In addition, the fungal toxin fumonisin B1 only marginally affected the viability of protoplasts from the *etr1-1* mutant and presence of the *ein2-1* mutation reduced cell death in the accelerated cell death 5 (*acd5*) mutant, supporting a role for ethylene in the regulation of programmed cell death (Asai et al., 2000; Greenberg et al., 2000). On the contrary, *ein2-1* showed markedly enhanced susceptibility to two different strains of the necrotrophic fungus *Botrytis cinerea* (Thomma et al., 1999). Conversely, constitutive expression of *ERF1* in *Arabidopsis* is sufficient to confer resistance to both *Botrytis cinerea* and *Pseudomonas cucumerina* (Berrocal-Lobo et al., 2002). In conclusion, these data provide strong support to the notion that ethylene can play a balanced role in mounting disease resistance, the outcome of which is dependent on the nature of the pathogen. This apparent discrepancy among the roles of ethylene in different plant-pathogen interactions may be reconciled by the different infection mechanisms of different pathogens, and by the fact that ethylene is not only involved in pathogen response, but is a hormone implicated in many general aspects of plant development including senescence, cell death, and ripening (Abeles et al., 1992). Therefore, the detrimental effect of ethylene in some plant-pathogen interactions may be an indirect consequence of the involvement of the hormone in the above-mentioned developmental processes.

Besides its involvement in pathogen infection, ethylene is also implicated in response to abiotic stresses. An enhanced ethylene emanation is one of the earliest responses to ozone stress (Vahala et al., 1998; Moeder et al., 2002). The expression of *ACS6* in *Arabidopsis* is activated within 30 min. after the onset of ozone exposure (Vahala et al., 1998). Exposure to ozone leads to a rapid oxidative burst, which evokes a local cell death response similar to that caused by the hypersensitive response upon pathogen infection

(Pell et al., 1997). An ozone-insensitive mutant, *rcd1* (radical-induced cell death), has been shown to have a higher susceptibility to the oxidative burst (Overmyer et al., 2000). The prolonged cell death response was suppressed in *rcd1* by mutations in *EIN2*, suggesting that ethylene signalling is required for cell death (Overmyer et al., 2000). It is suggested that ethylene is involved in the regulation of cell death by amplifying ROS production, which is responsible for the execution and spreading of cell death. Compared with the two other hormones involved in responses to abiotic stresses and pathogen defence, JA and SA, ethylene is involved in the early responses whereas JA and SA may control more prolonged effects.

1.5 TRANSCRIPTIONAL REGULATION OF ETHYLENE RESPONSE

The plant hormone ethylene affects many different aspects of plant development. One hypothesis for the diversity of physiological and biochemical responses to ethylene is that ethylene regulates the expression of a myriad of genes. A few years ago, Lincoln and Fisher (1988) investigated the mechanism of action of ethylene by analyzing the expression of ethylene-inducible genes isolated from tomato (Lincoln and Fischer, 1988). Their results indicated that there are multiple mechanisms for the regulation of gene expression by ethylene and that the expression of different classes of genes is regulated by ethylene in a fairly unique fashion. For certain genes ethylene affected transcriptional control, while for others it affected post-transcriptional processes. Furthermore, by measuring gene expression as a function of ethylene concentration, they demonstrated that the tested genes displayed a unique, narrow dose response curve in response to exogenous ethylene. In addition, the transcriptional activation of some genes was organ specific, while for others it was not. Finally, ethylene was capable of inducing changes in plant physiology by rapidly altering patterns of gene transcription. The molecular basis for this diversity will be better understood when the DNA sequences and cellular factors that regulate ethylene-inducible gene expression are isolated and analyzed. The uncovering of the transcriptional cascade controlling ethylene response, involving two families of transcription factors (see 2.2.5) represented an important step in unravelling the different players by which ethylene regulates gene expression, although this is not sufficient to explain the diversity of plant responses to the hormone.

Various targeted gene expression studies have identified different ethylene-regulated genes in different processes and in different tissue types.

Through differential screening techniques a number of early ethylene-regulated genes were isolated in etiolated seedlings and during tomato ripening (Zegzouti et al., 1999;

Trentmann, 2000). In the first analysis an ethylene-regulated nuclear-localized protein, designated ERN1, was identified, its expression was suppressed by ethylene (Trentmann, 2000). The latter analysis yielded a number of genes involved in fruit ripening. Their study showed that the predicted proteins encoded by the isolated genes involved a wide diversity of functions indicating the complexity of cellular responses to ethylene. Interestingly, ethylene-dependent changes in mRNA accumulation occurred rapidly (15 min.) for most of the isolated clones (Zegzouti et al., 1999).

In addition, changes in the expression patterns of 2.375 selected genes were examined simultaneously by cDNA microarray analysis in *Arabidopsis* after inoculation with an incompatible fungal pathogen or treatment with the defence-related signalling molecules salicylic acid (SA), methyl jasmonate (MJ), or ethylene (Schenk et al., 2000). Their results demonstrated that considerable interaction occurs among the different defence signalling pathways, notably between the SA and MJ pathways. Fifty percent of the genes induced by ethylene treatment were also induced by MJ treatment.

Another study focused on transcriptional profiling of genes in response to wounding and demonstrated that besides the reported wounding-mediated ethylene biosynthesis, crosstalk may also occur between wounding and the ethylene signalling pathway at the level of transcriptional regulation (Cheong et al., 2002).

Recently, Alonso et al. (2003b) reported the use of Affymetrix gene expression arrays to examine the RNA levels of more than 22.000 genes in response to ethylene. Although their results are not yet published, they demonstrated that four genes that encode proteins with two plant-specific DNA binding domains, AP2 and B3, were found to be ethylene-inducible (Alonso et al., 2003b). These genes were named *ETHYLENE RESPONSE DNA BINDING FACTORS (EDF).*

Finally, ethylene-regulated gene expression was investigated in *Arabidopsis* leaves using a cDNA microarray containing about 6000 unique genes (Van Zhong and Burns, 2003). In this study, the focus was put on late-term (24h) ethylene regulation and their results were compared to *etr1-1* and *ctr1-1*.

Until now, no kinetic analysis of the transcriptional cascade to very early responses to ethylene has been performed.

1.6 TRANSCRIPT PROFILING TECHNIQUES: PRO'S AND CON'S

Genome-wide expression analysis is a valuable tool to help determining the functions of genes and their spatial and temporal expression patterns, as well as elucidating the genetic networks in which they participate. Microarrays have been used successfully in a

number of studies addressing diverse questions and are rapidly becoming the standard tool for genome-wide expression analysis. The two microarray-based technologies – cDNA and oligonucleotide microarrays – have proven to be powerful for genome-wide expression analysis in a wide range of organisms, including plants. The strength of their success lies in the massive parallel nature of the analysis, which allows up to tens of thousands of genes to be analyzed simultaneously. Nowadays, microarrays comprising the complete gene set of *Arabidopsis* are available (http://www.york.ac.uk/res/garnet/projects.htm , http://www.catma.org/). Furthermore, large data sets from independent experiments can be combined together in a single database, which allows gene expression profiles from either different samples or samples obtained using different treatments to be compared and analyzed together (Brazma et al., 2001) (http://www.mged.org/miame). Despite its strengths, the current microarray technology still has limitations. Their utility is currently restricted to a small number of organisms, because only the fraction of the genes for which either the DNA sequence or a cDNA clone is available can be surveyed. Abundant messengers are over-represented in cDNA libraries, whereas rarely expressed genes are often missing, therefore many important genes will be overlooked by these microarrays. A second disadvantage for the use of microarrays is the difficulty in distinguishing among homologous genes. Transcripts from genes that exhibit a high degree of sequence homology will often cross-hybridize. For *Arabidopsis* it is described that a large fraction of the genes is duplicated. Technical improvements like using gene-specific tags could overcome this limitation as is largely the case in Affymetrix and CATMA chips. Finally, another current drawback of the method is its limited sensitivity of hybridization.

Alternative methods for genome-wide expression analysis, based on sequencing or PCR amplification of transcript tags, overcome some of the limitations of microarrays (Breyne and Zabeau, 2001). cDNA-AFLP has been applied with success for gene discovery in particular biological processes (Durrant et al., 2000; Qin et al., 2000; van der Biezen et al., 2000). Moreover, an improved cDNA-AFLP method has been shown to be an efficient tool for quantitative genome-wide transcription analysis and a valid alternative to microarrays (Breyne et al., 2003). One of the improvements is that only one unique fragment is obtained from each cDNA. In addition, by increasing the stringency of the PCR amplification step, one can vary the degree of fractionation and, hence, increase the sensitivity of the analysis. In this way, even scarcely expressed genes can be detected. Because the method is independent of sequence information, it allows expression analysis in any species and both known and unknown genes can be analyzed. The disadvantage of this method is that generating a global view of gene expression patterns involves a time-

consuming series of PCR and sequencing reactions. Especially due to the latter and the availability of microarrays for *Arabidopsis*, we first performed a study of early ethylene expression analysis by using the cDNA-AFLP method. This study gave us an idea about the value of the chosen time-points of treatment and provided insights to establish the setup for a more extensive microarray experiment. As we made use of both techniques, we could perform a comparative analysis of both methods. In the following chapter (Chapter 2), the characterization of novel ethylene regulated genes using cDNA-AFLP is described and discussed. The analysis of early ethylene-regulated transcription by microarray analysis is presented in chapter 4. Part 4.6 of this chapter evaluates both methods and illustrates a comparative analysis.

1.7 Scope of this thesis

The subject of this study is situated in the field of ethylene signalling in *Arabidopsis thaliana*. Ethylene is a potent modulator of plant growth and development. To understand the role of ethylene in plant growth, it is important to know how gaseous hormone is synthesized, how its production is regulated, and how its signal is transduced. The aim of this work was to gain knowledge particularly at the level of ethylene signalling. To this end, two different approaches were followed.

One way to obtain a better insight in ethylene signalling is to examine ethylene-regulated transcription. Although a tremendous progress has been made elucidating the mechanisms of synthesis, perception, and transduction of the ethylene signal, little is known about the genes that are transcriptionally regulated in response to ethylene. The identification of early ethylene-regulated genes is a strategy to gain further understanding of the molecular mechanisms of ethylene action. In an attempt to identify novel genes implicated in ethylene response, we screened for early ethylene-responsive genes in *Arabidopsis* using a cDNA-AFLP-transcript profiling approach and microarrays. An extensive kinetic analysis of the transcriptional cascade in response to ethylene would allow a comprehensive mapping of the interaction between ethylene and other pathways at the genomic level, and identification of novel genes involved in ethylene signalling. A comparison of genetic interaction maps with the hierarchy of gene expression based on transcript profiling and other genomic technologies may allow a more representative view of the integration of a hormone with other signals within the cell. The results are described in Part I (Chapter 2-4) of this manuscript.

The second approach to gain new insight in the mechanism of ethylene signal transduction is by isolating ethylene-response mutants. Most of the ethylene mutants have been isolated on the basis of defects in the ethylene-mediated triple response of dark-grown seedlings. Although this screening approach has led to substantial insight in the mechanisms of ethylene signalling, it has almost reached its saturation. Therefore, alternative mutant screens are probably needed to uncover additional components in ethylene signalling. By using a new screening method developed in our lab (Smalle et al., 1997), we isolated ethylene response mutants displaying enhanced ethylene responses (*eer*). They were identified based on an exaggerated hypocotyl elongation in the light on low nutrient medium (LNM) containing 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene essentially indicating hypersensitivity to ethylene. This approach resulted in the isolation of *eer2*, which displays significant hypocotyl elongation in the

presence of a 50-fold lower concentration of ACC as compared to the wild type. Part II (Chapter 5) of this manuscript will focus on the physiological characterization of the *eer2*-mutant and some additional enhanced ethylene response mutants recently isolated by screening for a similar hypersensitive response (Chapter 6).

1.8 REFERENCES

Abel, S., Nguyen, M.D., Chow, W., and Theologis, A. (1995). ACS4, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in Arabidopsis thaliana. Structural characterization, expression in Escherichia coli, and expression characteristics in response to auxin [corrected]. J Biol Chem **270,** 19093-19099.

Abeles, F.B., Morgan, P.W., and Saltveit, M.E. (1992). Ethylene in Plant Biology, 2nd Edition. (San Diego: Academic Press).

Achard, P., Vriezen, W.H., Van Der Straeten, D., and Harberd, N.P. (2003). Ethylene Regulates Arabidopsis Development via the Modulation of DELLA Protein Growth Repressor Function. Plant Cell.

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S., and Ecker, J.R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. Science **284,** 2148-2152.

Alonso, J.M., Stepanova, A.N., Solano, R., Wisman, E., Ferrari, S., Ausubel, F.M., and Ecker, J.R. (2003a). Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in Arabidopsis. Proc Natl Acad Sci U S A **100,** 2992-2997.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., and Ecker, J.R. (2003b). Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science **301,** 653-657.

Aravind, L., and Ponting, C.P. (1997). The GAF domain: an evolutionary link between diverse phototransducing proteins. Trends Biochem Sci **22,** 458-459.

Arteca, J.M., and Arteca, R.N. (1999). A multi-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase (ACS6) in mature Arabidopsis leaves. Plant Mol Biol **39,** 209-219.

Asai, T., Stone, J.M., Heard, J.E., Kovtun, Y., Yorgey, P., Sheen, J., and Ausubel, F.M. (2000). Fumonisin B1-induced cell death in arabidopsis protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways. Plant Cell **12,** 1823-1836.

Barry, C.S., Blume, B., Bouzayen, M., Cooper, W., Hamilton, A.J., and Grierson, D. (1996). Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. Plant J **9,** 525-535.

Beaudoin, N., Serizet, C., Gosti, F., and Giraudat, J. (2000). Interactions between abscisic acid and ethylene signaling cascades. Plant Cell **12,** 1103-1115.

Bennett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schulz, B., and Feldmann, K.A. (1996). Arabidopsis AUX1 gene: a permease-like regulator of root gravitropism. Science **273,** 948-950.

Bent, A.F., Innes, R.W., Ecker, J.R., and Staskawicz, B.J. (1992). Disease development in ethylene-insensitive Arabidopsis thaliana infected with virulent and avirulent Pseudomonas and Xanthomonas pathogens. Mol Plant Microbe Interact **5,** 372-378.

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. Plant J **29,** 23-32.

Bleecker, A.B., and Kende, H. (2000). Ethylene: a gaseous signal molecule in plants. Annu Rev Cell Dev Biol **16,** 1-18.

Blumer, K.J., and Johnson, G.L. (1994). Diversity in function and regulation of MAP kinase pathways. Trends Biochem Sci **19,** 236-240.

Botella, J.R., Arteca, R.N., and Frangos, J.A. (1995). A mechanical strain-induced 1-aminocyclopropane-1-carboxylic acid synthase gene. Proc Natl Acad Sci U S A **92,** 1595-1598.

Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C.A., Causton, H.C., Gaasterland, T., Glenisson, P., Holstege, F.C., Kim, I.F., Markowitz, V., Matese, J.C., Parkinson, H., Robinson, A., Sarkans, U., Schulze-Kremer, S., Stewart, J., Taylor, R., Vilo, J., and Vingron, M. (2001). Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet **29,** 365-371.

Breyne, P., and Zabeau, M. (2001). Genome-wide expression analysis of plant cell cycle modulated genes. Curr Opin Plant Biol **4,** 136-142.

Breyne, P., Dreesen, R., Cannoot, B., Rombaut, D., Vandepoele, K., Rombauts, S., Vanderhaeghen, R., Inze, D., and Zabeau, M. (2003). Quantitative cDNA-AFLP analysis for genome-wide expression studies. Mol Genet Genomics **269,** 173-179.

Buck, V., Quinn, J., Soto Pino, T., Martin, H., Saldanha, J., Makino, K., Morgan, B.A., and Millar, J.B. (2001). Peroxide sensors for the fission yeast stress-activated mitogen-activated protein kinase pathway. Mol Biol Cell **12,** 407-419.

Cancel, J.D., and Larsen, P.B. (2002). Loss-of-function mutations in the ethylene receptor ETR1 cause enhanced sensitivity and exaggerated response to ethylene in Arabidopsis. Plant Physiol **129,** 1557-1567.

Cary, A.J., Liu, W., and Howell, S.H. (1995). Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyl elongation in Arabidopsis thaliana seedlings. Plant Physiol **107,** 1075-1082.

Chae, H.S., Faure, F., and Kieber, J.J. (2003). The eto1, eto2, and eto3 Mutations and Cytokinin Treatment Increase Ethylene Biosynthesis in Arabidopsis by Increasing the Stability of ACS Protein. Plant Cell **15,** 545-559.

Chang, C., and Stadler, R. (2001). Ethylene hormone receptor action in Arabidopsis. Bioessays **23,** 619-627.

Chang, C., Kwok, S.F., Bleecker, A.B., and Meyerowitz, E.M. (1993). Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. Science **262,** 539-544.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R. (1997). Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell **89,** 1133-1144.

Chen, Q.G., and Bleecker, A.B. (1995). Analysis of ethylene signal-transduction kinetics associated with seedling-growth response and chitinase induction in wild-type and mutant arabidopsis. Plant Physiol **108,** 597-607.

Chen, Y.F., Randlett, M.D., Findell, J.L., and Schaller, G.E. (2002). Localization of the ethylene receptor ETR1 to the endoplasmic reticulum of Arabidopsis. J Biol Chem **277,** 19861-19866.

Cheong, Y.H., Chang, H.S., Gupta, R., Wang, X., Zhu, T., and Luan, S. (2002). Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. Plant Physiol **129,** 661-677.

Clark, K.L., Larsen, P.B., Wang, X., and Chang, C. (1998). Association of the Arabidopsis CTR1 Raf-like kinase with the ETR1 and ERS ethylene receptors. Proc Natl Acad Sci U S A **95,** 5401-5406.

Cosgrove, D.J., Gilroy, S., Kao, T.-h., Ma, H., and Schultz, J.C. (2000). Plant signaling 2000: Cross talk among geneticists, physiologists, and ecologists. Plant Physiol. **124,** 499-506.

Durrant, W.E., Rowland, O., Piedras, P., Hammond-Kosack, K.E., and Jones, J.D. (2000). cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. Plant Cell **12,** 963-977.

Fu, X., and Harberd, N.P. (2003). Auxin promotes Arabidopsis root growth by modulating gibberellin response. Nature **421,** 740-743.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H., and Ohme-Takagi, M. (2000). Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. Plant Cell **12,** 393-404.

Fujita, H., and Syono, K. (1996). Genetic analysis of the effects of polar auxin transport inhibitors on root growth in Arabidopsis thaliana. Plant Cell Physiol **37,** 1094-1101.

Gao, Z., Chen, Y.F., Randlett, M.D., Zhao, X.C., Findell, J.L., Kieber, J.J., and Schaller, G.E. (2003). Localization of the Raf-like kinase CTR1 to the endoplasmic reticulum of

Arabidopsis through participation in ethylene receptor signaling complexes. J Biol Chem **278,** 34725-34732.

Ghassemian, M., Nambara, E., Cutler, S., Kawaide, H., Kamiya, Y., and McCourt, P. (2000). Regulation of abscisic acid signaling by the ethylene response pathway in Arabidopsis. Plant Cell **12,** 1117-1126.

Glazebrook, J. (1999). Genes controlling expression of defense responses in Arabidopsis. Curr Opin Plant Biol **2,** 280-286.

Gomez-Lim, M.A., Valdes-Lopez, V., Cruz-Hernandez, A., and Saucedo-Arias, L.J. (1993). Isolation and characterization of a gene involved in ethylene biosynthesis from Arabidopsis thaliana. Gene **134,** 217-221.

Greenberg, J.T., Silverman, F.P., and Liang, H. (2000). Uncoupling salicylic acid-dependent cell death and defense-related responses from disease resistance in the Arabidopsis mutant acd5. Genetics **156,** 341-350.

Guo, H., and Ecker, J.R. (2003). Plant responses to ethylene gas are mediated by SCF(EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. Cell **115,** 667-677.

Guzman, P., and Ecker, J.R. (1990). Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell **2,** 513-523.

Hall, A.E., Chen, Q.G., Findell, J.L., Schaller, G.E., and Bleecker, A.B. (1999). The relationship between ethylene binding and dominant insensitivity conferred by mutant forms of the ETR1 ethylene receptor. Plant Physiol **121,** 291-300.

Hall, A.E., Findell, J.L., Schaller, G.E., Sisler, E.C., and Bleecker, A.B. (2000). Ethylene perception by the ERS1 protein in Arabidopsis. Plant Physiol **123,** 1449-1458.

Harper, R.M., Stowe-Evans, E.L., Luesse, D.R., Muto, H., Tatematsu, K., Watahiki, M.K., Yamamoto, K., and Liscum, E. (2000). The NPH4 locus encodes the auxin response factor ARF7, a conditional regulator of differential growth in aerial Arabidopsis tissue. Plant Cell **12,** 757-770.

Hirayama, T., Kieber, J.J., Hirayama, N., Kogan, M., Guzman, P., Nourizadeh, S., Alonso, J.M., Dailey, W.P., Dancis, A., and Ecker, J.R. (1999). RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. Cell **97,** 383-393.

Hua, J., and Meyerowitz, E.M. (1998). Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. Cell **94,** 261-271.

Hua, J., Chang, C., Sun, Q., and Meyerowitz, E.M. (1995). Ethylene insensitivity conferred by Arabidopsis ERS gene. Science **269,** 1712-1714.

Hua, J., Sakai, H., Nourizadeh, S., Chen, Q.G., Bleecker, A.B., Ecker, J.R., and Meyerowitz, E.M. (1998). EIN4 and ERS2 are members of the putative ethylene receptor gene family in Arabidopsis. Plant Cell **10,** 1321-1332.

Huang, Y., Li, H., Hutchison, C.E., Laskey, J., and Kieber, J.J. (2003). Biochemical and functional analysis of CTR1, a protein kinase that negatively regulates ethylene signaling in Arabidopsis. Plant J **33,** 221-233.

Hwang, I., Chen, H.C., and Sheen, J. (2002). Two-component signal transduction pathways in Arabidopsis. Plant Physiol **129,** 500-515.

Johnson, P.R., and Ecker, J.R. (1998). The ethylene gas signal transduction pathway: a molecular perspective. Annu Rev Genet **32,** 227-254.

Kende, H. (1993). Ethylene biosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. **44,** 283–307.

Kepinski, S., and Leyser, O. (2002). Ubiquitination and auxin signaling: a degrading story. Plant Cell **14 Suppl,** S81-95.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R. (1993). CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases. Cell **72,** 427-441.

Kim, W.T., and Yang, S.F. (1994). Structure and expression of cDNAs encoding 1-aminocyclopropane-1-carboxylate oxidase homologs isolated from excised mung bean hypocotyls. Planta **194,** 223-229.

Larsen, P.B., and Chang, C. (2001). The Arabidopsis eer1 Mutant Has Enhanced Ethylene Responses in the Hypocotyl and Stem. Plant Physiol **125,** 1061-1073.

Larsen, P.B., and Cancel, J.D. (2003). Enhanced ethylene responsiveness in the Arabidopsis eer1 mutant results from a loss-of-function mutation in the protein phosphatase 2A A regulatory subunit, RCN1. Plant J **34,** 709-718.

Lasserre, E., Bouquin, T., Hernandez, J.A., Bull, J., Pech, J.C., and Balague, C. (1996). Structure and expression of three genes encoding ACC oxidase homologs from melon (Cucumis melo L.). Mol Gen Genet **251,** 81-90.

Lee, S., Cheng, H., King, K.E., Wang, W., He, Y., Hussain, A., Lo, J., Harberd, N.P., and Peng, J. (2002). Gibberellin regulates Arabidopsis seed germination via RGL2, a GAI/RGA-like gene whose expression is up-regulated following imbibition. Genes Dev **16,** 646-658.

Lehman, A., Black, R., and Ecker, J.R. (1996). HOOKLESS1, an ethylene response gene, is required for differential cell elongation in the Arabidopsis hypocotyl. Cell **85,** 183-194.

Leon, P., and Sheen, J. (2003). Sugar and hormone connections. Trends Plant Sci **8,** 110-116.

Liang, X., Shen, N.F., and Theologis, A. (1996). Li(+)-regulated 1-aminocyclopropane-1-carboxylate synthase gene expression in Arabidopsis thaliana. Plant J **10,** 1027-1036.

Liang, X., Abel, S., Keller, J.A., Shen, N.F., and Theologis, A. (1992). The 1-aminocyclopropane-1-carboxylate synthase gene family of Arabidopsis thaliana. Proc Natl Acad Sci U S A **89,** 11046-11050.

Liang, X., Oono, Y., Shen, N.F., Kohler, C., Li, K., Scolnik, P.A., and Theologis, A. (1995). Characterization of two members (ACS1 and ACS3) of the 1-aminocyclopropane-1-carboxylate synthase gene family of Arabidopsis thaliana. Gene **167,** 17-24.

Lincoln, J.E., and Fischer, R.L. (1988). Diverse mechanisms for the regulation of ethylene-inducible gene expression. Mol Gen Genet **212,** 71-75.

Marchant, A., Kargul, J., May, S.T., Muller, P., Delbarre, A., Perrot-Rechenmann, C., and Bennett, M.J. (1999). AUX1 regulates root gravitropism in Arabidopsis by facilitating auxin uptake within root apical tissues. Embo J **18,** 2066-2073.

McGinnis, K.M., Thomas, S.G., Soule, J.D., Strader, L.C., Zale, J.M., Sun, T.P., and Steber, C.M. (2003). The Arabidopsis SLEEPY1 Gene Encodes a Putative F-Box Subunit of an SCF E3 Ubiquitin Ligase. Plant Cell **15,** 1120-1130.

Mehta, P.K., Hale, T.I., and Christen, P. (1993). Aminotransferases: demonstration of homology and division into evolutionary subgroups. Eur J Biochem **214,** 549-561.

Moeder, W., Barry, C.S., Tauriainen, A.A., Betz, C., Tuomainen, J., Utriainen, M., Grierson, D., Sandermann, H., Langebartels, C., and Kangasjarvi, J. (2002). Ethylene synthesis regulated by biphasic induction of 1-aminocyclopropane-1-carboxylic acid synthase and 1-aminocyclopropane-1-carboxylic acid oxidase genes is required for hydrogen peroxide accumulation and cell death in ozone-exposed tomato. Plant Physiol **130,** 1918-1926.

Muller-Dieckmann, H.J., Grantz, A.A., and Kim, S.H. (1999). The structure of the signal receiver domain of the Arabidopsis thaliana ethylene receptor ETR1. Structure Fold. Des. **7,** 1547-1556.

Nadeau, J.A., Zhang, X.S., Nair, H., and O'Neill, S.D. (1993). Temporal and spatial regulation of 1-aminocyclopropane-1-carboxylate oxidase in the pollination-induced senescence of orchid flowers. Plant Physiol **103,** 31-39.

Oh, S.A., Park, J.H., Lee, G.I., Paek, K.H., Park, S.K., and Nam, H.G. (1997). Identification of three genetic loci controlling leaf senescence in Arabidopsis thaliana. Plant J **12,** 527-535.

Ohme-Takagi, M., and Shinshi, H. (1995). Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. Plant Cell **7,** 173-182.

Ouaked, F., Rozhon, W., Lecourieux, D., and Hirt, H. (2003). A MAPK pathway mediates ethylene signaling in plants. Embo J **22,** 1282-1288.

Overmyer, K., Tuominen, H., Kettunen, R., Betz, C., Langebartels, C., Sandermann, H., Jr., and Kangasjarvi, J. (2000). Ozone-sensitive arabidopsis rcd1 mutant reveals opposite roles for ethylene and jasmonate signaling pathways in regulating superoxide-dependent cell death. Plant Cell **12,** 1849-1862.

Pell, E.J., Schlagnhaufer, C.D., and Arteca, R.N. (1997). Ozone-induced oxidative stress: Mechanisms of action and reaction. Physiol. Plant. **100,** 264-273.

Peng, J., Carol, P., Richards, D.E., King, K.E., Cowling, R.J., Murphy, G.P., and Harberd, N.P. (1997). The Arabidopsis GAI gene defines a signaling pathway that negatively regulates gibberellin responses. Genes Dev **11,** 3194-3205.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell **10,** 2103-2113.

Pitts, R.J., Cernac, A., and Estelle, M. (1998). Auxin and ethylene promote root hair elongation in Arabidopsis. Plant J **16,** 553-560.

Posas, F., Wurgler-Murphy, S.M., Maeda, T., Witten, E.A., Thai, T.C., and Saito, H. (1996). Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. Cell **86,** 865-875.

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C., and Genschik, P. (2003). EIN3-dependent regulation of plant ethylene hormone signaling by two arabidopsis F box proteins: EBF1 and EBF2. Cell **115,** 679-689.

Qin, L., Overmars, H., Helder, J., Popeijus, H., van der Voort, J.R., Groenink, W., van Koert, P., Schots, A., Bakker, J., and Smant, G. (2000). An efficient cDNA-AFLP-based strategy for the identification of putative pathogenicity factors from the potato cyst nematode Globodera rostochiensis. Mol Plant Microbe Interact **13,** 830-836.

Rahman, A., Amakawa, T., Goto, N., and Tsurumi, S. (2001). Auxin is a positive regulator for ethylene-mediated response in the growth of Arabidopsis roots. Plant Cell Physiol **42,** 301-307.

Ravanel, S., Gakiere, B., Job, D., and Douce, R. (1998). The specific features of methionine biosynthesis and metabolism in plants. Proc Natl Acad Sci U S A **95,** 7805-7812.

Raz, V., and Ecker, J.R. (1999). Regulation of differential growth in the apical hook of Arabidopsis. Development **126,** 3661-3668.

Rodrigues-Pousada, R.A., De Rycke, R., Dedonder, A., Van Caeneghem, W., Engler, G., Van Montagu, M., and Van Der Straeten, D. (1993). The Arabidopsis 1-Aminocyclopropane-1-Carboxylate Synthase Gene 1 Is Expressed during Early Development. Plant Cell **5,** 897-911.

Rodriguez, F.I., Esch, J.J., Hall, A.E., Binder, B.M., Schaller, G.E., and Bleecker, A.B. (1999). A copper cofactor for the ethylene receptor ETR1 from Arabidopsis. Science **283,** 996-998.

Roman, G., and Ecker, J.R. (1995). Genetic analysis of a seedling stress response to ethylene in Arabidopsis. Philos Trans R Soc Lond B Biol Sci **350,** 75-81.

Roman, G., Lubarsky, B., Kieber, J.J., Rothenberg, M., and Ecker, J.R. (1995). Genetic analysis of ethylene signal transduction in Arabidopsis thaliana: five novel mutant loci integrated into a stress response pathway. Genetics **139,** 1393-1409.

Saibo, N.J., Vriezen, W.H., Beemster, G.T., and Van Der Straeten, D. (2003). Growth and stomata development of Arabidopsis hypocotyls are controlled by gibberellins and modulated by ethylene and auxins. Plant J **33,** 989-1000.

Sakai, H., Hua, J., Chen, Q.G., Chang, C., Medrano, L.J., Bleecker, A.B., and Meyerowitz, E.M. (1998). ETR2 is an ETR1-like gene involved in ethylene signaling in Arabidopsis. Proc Natl Acad Sci U S A **95,** 5812-5817.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., and Coupland, G. (2000). Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science **288,** 1613-1616.

Schaller, G.E., and Bleecker, A.B. (1995). Ethylene-binding sites generated in yeast expressing the Arabidopsis ETR1 gene. Science **270,** 1809-1811.

Schaller, G.E., and Kieber, J.J. (2002). Ethylene. (The Arabidopsis Book, eds. C.R. Somerville and E.M. Meyerowitz, American Society of Plant Biologists, Rockville, MD).

Schaller, G.E., Ladd, A.N., Lanahan, M.B., Spanbauer, J.M., and Bleecker, A.B. (1995). The ethylene response mediator ETR1 from Arabidopsis forms a disulfide-linked dimer. J Biol Chem **270,** 12526-12530.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., and Manners, J.M. (2000). Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proc Natl Acad Sci U S A **97,** 11655-11660.

Shieh, J.C., Wilkinson, M.G., Buck, V., Morgan, B.A., Makino, K., and Millar, J.B. (1997). The Mcs4 response regulator coordinately controls the stress-activated Wak1-Wis1-Sty1 MAP kinase pathway and fission yeast cell cycle. Genes Dev **11,** 1008-1022.

Silverstone, A.L., Jung, H.S., Dill, A., Kawaide, H., Kamiya, Y., and Sun, T.P. (2001). Repressing a repressor: gibberellin-induced rapid reduction of the RGA protein in Arabidopsis. Plant Cell **13,** 1555-1566.

Smalle, J., Haegman, M., Kurepa, J., Van Montagu, M., and Van Der Straeten, D. (1997). Ethylene can stimulate Arabidopsis hypocotyl elongation in the light. Proc Natl Acad Sci U S A **94,** 2756-2761.

Smalle, J., Kurepa, J., Yang, P., Babiychuk, E., Kushnir, S., Durski, A., and Vierstra, R.D. (2002). Cytokinin growth responses in Arabidopsis involve the 26S proteasome subunit RPN12. Plant Cell **14,** 17-32.

Smalle, J., Kurepa, J., Yang, P., Emborg, T.J., Babiychuk, E., Kushnir, S., and Vierstra, R.D. (2003). The pleiotropic role of the 26S proteasome subunit RPN10 in Arabidopsis growth and development supports a substrate-specific function in abscisic acid signaling. Plant Cell **15,** 965-980.

Smith, J.M., and Arteca, R.N. (2000). Molecular control of ethylene production by cyanide in Arabidopsis thaliana. Physiol. Plant. **109,** 180-187.

Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R. (1998). Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev **12,** 3703-3714.

Stock, A.M., Robinson, V.L., and Goudreau, P.N. (2000). Two-component signal transduction. Annu Rev Biochem **69,** 183-215.

Su, W.P., and Howell, S.H. (1992). A single genetic-locus, ckr1, defines Arabidopsis mutants in which root-growth is resistant to low concentrations of cytokinin. Plant Physiol **99,** 1569-1574.

Suttle, J.C. (1988). Effect of ethylene treatment onn polar IAA transport, net IAA uptake and specific binding of N-1-naphthylphthalamic acid in tissues and microsomes isolated from etiolated pea epicotyls. Plant Physiol **88,** 795-799.

Tang, X., and Woodson, W.R. (1996). Temporal and Spatial Expression of 1-Aminocyclopropane-1-Carboxylate Oxidase mRNA following Pollination of Immature and Mature Petunia Flowers. Plant Physiol **112,** 503-511.

Tatsuki, M., and Mori, H. (2001). Phosphorylation of tomato 1-aminocyclopropane-1-carboxylic acid synthase, LE-ACS2, at the C-terminal region. J Biol Chem **276,** 28051-28057.

Thomine, S., Wang, R., Ward, J.M., Crawford, N.M., and Schroeder, J.I. (2000). Cadmium and iron transport by members of a plant metal transporter family in Arabidopsis with homology to Nramp genes. Proc Natl Acad Sci U S A **97,** 4991-4996.

Thomma, B.P., Eggermont, K., Tierens, K.F., and Broekaert, W.F. (1999). Requirement of functional ethylene-insensitive 2 gene for efficient resistance of Arabidopsis to infection by Botrytis cinerea. Plant Physiol **121,** 1093-1102.

Trentmann, S.M. (2000). ERN1, a novel ethylene-regulated nuclear protein of Arabidopsis. Plant Mol Biol **44,** 11-25.

Vahala, J., Schlagnhaufer, C.D., and Pell, E.J. (1998). Induction of an ACC synthase cDNA by ozone in light-grown Arabidopsis thaliana leaves. Physiol. Plant. **103,** 45-50.

van der Biezen, E.A., Juwana, H., Parker, J.E., and Jones, J.D. (2000). cDNA-AFLP display for the isolation of Peronospora parasitica genes expressed during infection in Arabidopsis thaliana. Mol Plant Microbe Interact **13,** 895-898.

Van der Straeten, D., Rodrigues-Pousada, R.A., Villarroel, R., Hanley, S., Goodman, H.M., and Van Montagu, M. (1992). Cloning, genetic mapping, and expression analysis of an Arabidopsis thaliana gene that encodes 1-aminocyclopropane-1-carboxylate synthase. Proc Natl Acad Sci U S A **89,** 9969-9973.

Van Zhong, G., and Burns, J.K. (2003). Profiling ethylene-regulated gene expression in Arabidopsis thaliana by microarray analysis. Plant Mol. Biol. **53,** 117-131.

Vandenbussche, F., Smalle, J., Le, J., Saibo, N.J., De Paepe, A., Chaerle, L., Tietz, O., Smets, R., Laarhoven, L.J., Harren, F.J., Van Onckelen, H., Palme, K., Verbelen, J.P., and Van Der Straeten, D. (2003). The Arabidopsis mutant alh1 illustrates a cross talk between ethylene and auxin. Plant Physiol **131,** 1228-1238.

Vogel, J.P., Woeste, K.E., Theologis, A., and Kieber, J.J. (1998b). Recessive and dominant mutations in the ethylene biosynthetic gene ACS5 of Arabidopsis confer cytokinin

insensitivity and ethylene overproduction, respectively. Proc Natl Acad Sci U S A **95,** 4766-4771.

Vogel, J.P., Schuerman, P., Woeste, K., Brandstatter, I., and Kieber, J.J. (1998). Isolation and characterization of Arabidopsis mutants defective in the induction of ethylene biosynthesis by cytokinin. Genetics **149,** 417-427.

Vogel, J.P., Schuerman, P., Woeste, K., Brandstatter, I., and Kieber, J.J. (1998a). Isolation and characterization of Arabidopsis mutants defective in the induction of ethylene biosynthesis by cytokinin. Genetics **149,** 417-427.

Vriezen W.H., Achard P., Harberd N.P., Van Der Straeten, D. (2004) Ethylene-mediated enhancement of apical hook formation in etiolated Arabidopsis thaliana seedlings is gibberellin dependent. Plant J **37** (in press)

Wang, K.L., Li, H., and Ecker, J.R. (2002). Ethylene biosynthesis and signaling networks. Plant Cell **14 Suppl,** S131-151.

Wang, W., Hall, A.E., O'Malley, R., and Bleecker, A.B. (2003). Canonical histidine kinase activity of the transmitter domain of the ETR1 ethylene receptor from Arabidopsis is not required for signal transmission. Proc Natl Acad Sci U S A **100,** 352-357.

Wen, C.K., and Chang, C. (2002). Arabidopsis RGL1 encodes a negative regulator of gibberellin responses. Plant Cell **14,** 87-100.

Woeste, K.E., and Kieber, J.J. (2000). A strong loss-of-function mutation in RAN1 results in constitutive activation of the ethylene response pathway as well as a rosette-lethal phenotype. Plant Cell **12,** 443-455.

Woeste, K.E., Ye, C., and Kieber, J.J. (1999). Two Arabidopsis mutants that overproduce ethylene are affected in the posttranscriptional regulation of 1-aminocyclopropane-1-carboxylic acid synthase. Plant Physiol **119,** 521-530.

Yamagami, T., Tsuchisaka, A., Yamada, K., Haddon, W.F., Harden, L.A., and Theologis, A. (2003). Biochemical characterization of the Arabidopsis ACSome. J Biol Chem.

Yanagisawa, S., Yoo, S.D., and Sheen, J. (2003). Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. Nature **425,** 521-525.

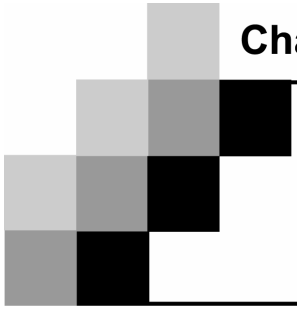
Yang, S.F., and Hoffman, N.E. (1984). Ethylene biosynthesis and its regulation in higher plants. Ann. Rev. Plant. Physiol. **35,** 155-189.

Zegzouti, H., Jones, B., Frasse, P., Marty, C., Maitre, B., Latch, A., Pech, J.C., and Bouzayen, M. (1999). Ethylene-regulated gene expression in tomato fruit: characterization of novel ethylene-responsive and ripening-related genes isolated by differential display. Plant J **18,** 589-600.

Zhou, L., Jang, J.C., Jones, T.L., and Sheen, J. (1998). Glucose and ethylene signal transduction crosstalk revealed by an Arabidopsis glucose-insensitive mutant. Proc Natl Acad Sci U S A **95,** 10294-10299.

PART I

Transcript Profiling of early ethylene responses in *Arabidopsis*.



Chapter 2

Transcript profiling by cDNA-AFLP reveals novel genes in response to ethylene in *Arabidopsis*

Chapter 2: Transcript profiling by cDNA-AFLP reveals novel genes in response to ethylene in *Arabidopsis*.

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2.1 SUMMARY

A cDNA-AFLP transcript profiling approach was used in order to gain further understanding of the molecular mechanisms of immediate ethylene action. Col-0 plants were treated with exogenous ethylene, 100ppb and 10ppm, for 6 different time-points ranging from 10 minutes until 6 hours. In order to isolate truly ethylene-responsive genes, the ethylene-insensitive mutant *ein2-1* and the constitutive ethylene-response mutant *ctr1-1* were analyzed in parallel. Forty-seven ethylene-responsive genes were isolated, falling in different classes of expression pattern and including a number of novel genes. The genes identified belong to diverse functional groups, including transcriptional and post-transcriptional regulators, protein degradation, cell wall metabolism, signal transduction components, defence response, metabolic processes, hormone signalling and finally photosynthesis. Analysis of ethylene responsiveness of 22 genes involved in the ubiquitin degradation pathway revealed that at least 4 genes, *UBP4*, *UBP23*, *UBP27*, and a putative *UBC* are induced by the hormone. Therefore, we propose that proteolytic degradation is part of ethylene signalling. Thus, this study provides new insights into the interaction between the ethylene signal transduction pathway and other pathways.

2.2 INTRODUCTION

The plant hormone ethylene is involved in many aspects of the plants life cycle, including seed germination, growth of stems, roots, and leaves; abscission, senescence, and fruit ripening. It also plays a role in responses to environmental stresses such as water deficit, mechanical wounding and pathogen attack (Abeles *et al.*, 1992; Johnson and Ecker, 1998). Over the past years, tremendous progress was made in elucidating the mechanisms of synthesis, perception, and transduction of the ethylene signal, albeit insufficient to explain the diversity of plant responses to the hormone. The ethylene signal transduction chain in *Arabidopsis* as presently understood consists of five partially functionally redundant receptors, which negatively regulate ethylene signalling (Bleecker *et al.*, 1988; Hua *et al.*, 1995; Hua and Meyerowitz, 1998; Hua *et al.*, 1998; Sakai *et al.*,

1998). Placed downstream of the ethylene receptors is CTR1, a Raf-like mitogen-activated protein kinase kinase kinase (MAPKKK) (Kieber et al., 1993). Direct evidence that a MAPK cascade is part of the ethylene signal transduction pathway in plants has been provided recently by Ouaked *et al*. (2003). *EIN2* acts downstream of *CTR1*; its amino terminal half encodes an integral membrane protein that exhibits significant similarity to the Nramp family of cation transporters (Alonso *et al.*, 1999). In response to ethylene, plants modulate the expression of specific genes at the transcriptional and post-transcriptional level (Koyama *et al*., 2001; Lincoln and Fischer, 1988; Zegzouti *et al.,* 1999). Responses downstream of EIN2 are modulated by a two-step cascade of transcriptional regulators involving two families of transcription factors, the EIN3 (ethylene insensitive)/EILs (EIN3-like) proteins and the ERFs (ethylene responsive factor) (Chao *et al.*, 1997). *EIN3* is a nuclear-localized DNA-binding protein belonging to a small multigene family in *Arabidopsis*. Two EIN3-like proteins, *EIL1* and *EIL2*, could complement a loss-of-function mutation in EIN3 and consequently are also involved in ethylene signal transduction. A search for target sequences in promoters for the EIN3/EIL transcription factors led to the identification of the primary ethylene response element (PERE) in the promotor of the *ERF1* gene (Sloan *et al.*, 1998). *ERF1* belongs to a large family of plant-specific transcription factors referred to as ERFs, which are capable of binding to a secondary ethylene response element, the GCC-box. This sequence was determined to be essential for the expression of several PR (pathogenesis-related) genes (Fujimoto *et al*., 2000; Zhou *et al*., 1997). Some ERF genes encode transcriptional activators, while others encode transcriptional repressors. The uncovering of this transcriptional cascade represents an important step in unravelling the different players by which ethylene regulates gene expression. However, a number of ethylene-response genes possess neither the PERE nor the GCC-box in their promoters. This observation indicates that other classes of transcription factors may also be involved in the transcriptional control of ethylene signalling. Identification of these proteins is crucial to complete the understanding of ethylene-mediated gene regulation. Various targeted gene expression studies have identified different ethylene-regulated genes in different processes and in different tissue types. Ethylene-response genes have been isolated using differential display in etiolated seedlings and during tomato ripening (Trentmann, 2000; Zegzouti *et al.,* 1999). Furthermore, recent studies using cDNA microarrays revealed genes regulated by jasmonic acid, ethylene, and upon infection with an avirulent pathogen, indicating a network of regulatory interactions and coordination during pathogen and wounding responses (Schenk *et al.*, 2000). Another study focused on transcriptional profiling of

genes in response to wounding and a number of genes involved in ethylene signalling were identified (Cheong *et al.*, 2002). Very recently, Van Zhong et al. described the profiling of ethylene-regulated gene expression in *Arabidopsis* by microarray analysis (Van Zhong and Burns, 2003). In this analysis only one time point (24h) was investigated; therefore restricted to later ethylene-response genes. In addition, their data was compared to the insensitive mutant *etr1-1* and the constitutive mutant *ctr1-1*, but no treatment was performed on the mutants.

Little is known about early ethylene-regulated genes in *Arabidopsis*, modulated shortly after ethylene exposure. Furthermore, the temporal cascade of gene expression in response to ethylene has not been analyzed in detail. To this end, we performed a study using the cDNA-AFLP transcript profiling technique. This sensitive and quantitative PCR-based technology allows to follow the kinetics of gene expression and to discriminate between genes belonging to multigene families (Breyne and Zabeau, 2001). In this study, 19 days old *Arabidopsis* plants were treated with two different concentrations of ethylene. At this stage of development ethylene plays a role in senescence (Park et al., 1998; Weaver et al., 1998). In addition, it is known that wounding, pathogen attack and different abiotic stresses induce ethylene production (Kende, 1993; Johnson and Ecker, 1998). Therefore, genes involved in ethylene-regulated stress responses are expected to come across our analysis. Moreover, this set-up allows exploring which vegetative processes are affected by ethylene.

Out of a first pilot cDNA-AFLP experiment, modulation of gene expression could be observed after the earliest time point of treatment (30 min.). In addition, 4 known ethylene-modulated genes were identified. In the focused cDNA-experiment, *Arabidopsis* wild type (Col-0) plants were treated with two different concentrations of ethylene, for a number of time-points ranging from 10 minutes until 6 hours. In order to isolate truly ethylene-responsive genes, the ethylene-insensitive mutant *ein2-1* (Alonso *et al.*, 1999) and the constitutive ethylene-response mutant *ctr1-1* (Kieber *et al.*, 1993) were included in the analysis. Out of 17 primer combinations covering approximately 5% of the *Arabidopsis* transcriptome, 47 ethylene-regulated transcript tags were isolated. Their expression profiles fell in distinct classes. An interesting group of genes displayed a rapid, transient increase of expression after 10 minutes of ethylene treatment. A second group of genes was specifically up-regulated after 1-2 h of ethylene treatment. For a third group of genes the mRNA level increased gradually upon ethylene exposure, whereas a fourth group displayed a decrease in mRNA in the presence of ethylene. A number of the isolated genes are novel in their responsiveness to ethylene.

2.3 RESULTS

2.3.1 Identification of different expression patterns of ethylene responsive genes by cDNA-AFLP analysis

2.3.1.1 Pilot cDNA-AFLP experiment

In a pilot cDNA-AFLP experiment, a first evaluation of differential expression in untreated and ethylene-treated wild type plants was performed. To that end, 45 primer combinations (selectivity +2/+1) were analyzed, covering about 15% of the genome, of wild type plants (Col-0) treated for 30 minutes and 3 hours with either 100ppb or 10ppm of ethylene. The experiment was done twice, thus two independent biological repeats were available for each sample. This analysis yielded 124 genes that were significantly (more than two-fold difference compared to WT for both independent experiments) changed in their expression by ethylene treatment. Ninety two genes were induced by ethylene, 32 genes were repressed by ethylene. Modulation of gene expression could be already observed after 30 minutes of treatment. In addition, 4 known ethylene-regulated genes were identified, all being expressed at elevated levels after 3h of treatment (Fig.1). Moreover, different expression patterns could be observed: genes up- or down regulated by all treatments, genes only regulated after the shorter or longer treatment, genes displaying an opposite pattern for the short compared to the longer treatment and finally genes which are ethylene-regulated only in the presence of either 100ppb or 10ppm (see table 1). The distribution of the transcript tags according to the function of these genes indicated that ethylene affected genes in many types of biological processes, including stress, senescence and defence, signalling, metabolism, protein degradation, and interaction with other signalling components as light and auxin.

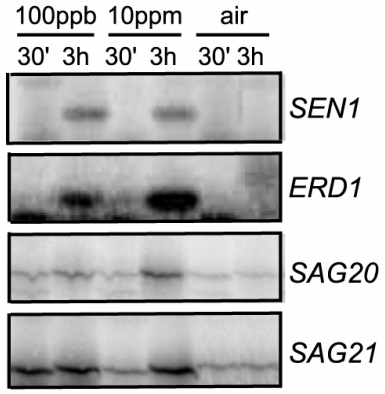


Figure 1: Induction pattern of 4 known ethylene-regulated genes in Col-0 treated for 30 minutes and 3 hours with 100ppb and 10ppm ethylene. The two control samples represent Col-0 treated with air for 30' and 3h respectively.
SEN: senescence-associated gene; *ERD*: early response to dehydratation; *SAG*: senescence-associated gene

Although this pilot-study resulted in some interesting observations, some drawbacks need to be considered for proper interpretation of these results. First of all, there is the lack of a control to rule out those genes that are only regulated by the circadian clock or by

mechanical stress during the treatment. This could be solved by adding ethylene mutants as a control, for instance an insensitive or constitutive mutant; or by treating the samples under continuous light conditions. In addition, only two data sets are available, representing an early and a later time-point. In order to interpret the statistical relevance of the results, one can either include more repeats, or additional data points. The latter has the advantage that the kinetic approach provides a clue to hierarchy in gene expression, which can help to build genetic interaction maps.

Table 1: Overview of isolated ethylene-regulated transcript tags. For all 124 transcript tags the primer combination, annotation, the length of the fragment, accession, and normalized data relative to wild type control for each time point is indicated. Green and red indicate more than two-fold higher repression or induction. The PCR-amplifications were performed with the *MseI* and *BstYI* primers-with either T or C as 3' nucleotide- with selective nucleotides (1=A, 2=C, 3=G, 4=T).

pimer combination	gene or protein	length(bp)	accession	Col-0 control	30 min. 100ppb	30min. 10ppm	3hr 100ppb	3hr 10ppm
BstT13-Mse3	no sequence	150		1	-1.3	-2.0	-1.7	-3.2
BstC12-Mse4	catalase	120	AY054663	1	-1.6	-2.2	-1.5	-2.3
BstT23-Mse3	AT3g25770/K13N2_9 mRNA,	280	AF141671	1	-2.9	-2.5	-1.1	-1.5
BstT23-Mse2	calmodulin-related protein (TCH2)gene	260	AF026473	1	-1.2	-2.0	-4.4	-4.6
BstC14-Mse2	cytochrome P450 CYP79FZ	210	AF370512	1	-1.3	-2.2	-3.5	-5.8
BstT23-Mse2	unknown protein AAC62866	160	AC002535	1	-2.0	-2.1	-2.8	-4.0
BstT21-Mse2	psbQ-gene, strong similarity to photosystemII-oxygen-evolving protein	110	BE038230	1	-7.5	-5.1	-3.7	-4.7
BstC44-Mse2	unknown protein F12K2_13	80	AY058862	1	-2.4	-1.8	-2.7	-2.8
BstC12-Mse3	between proteins	250	AC009398	1	-1.9	-1.0	-2.2	-1.5
BstC12-Mse3	unknown protein (AT4g32340) mRNA	90	AY039988	1	1.3	-1.4	-2.2	-1.7
BstC44-Mse21	animal hits	110		1	-2.0	-1.1	-4.2	-1.4
BstC12-Mse3	no sequence	80		1	1.5	1.1	-2.2	-1.4
BstC33-Mse33	no hits found	80		1	1.1	1.9	-2.3	-1.7
BstT33-Mse3	cytochrome P450 homolog-flovonoid 3'-5' hydroxylase-like protein	240	AL161533	1	-1.2	-1.8	-4.0	-5.1
BstC44-Mse2	putative-3-glyceraldehyde-3-phosphate dehydrogenase	250	AY039961	1	-1.6	-1.4	-2.6	-3.0
BstT33-Mse1	putative dUTP pyrophosphatase	200	AF370334	1	-1.2	1.0	-3.1	-3.8
BstC33-Mse34	unknown protein	80	AAC18935	1	1.2	1.1	-2.9	-3.4
BstC11-Mse1	similar to integral membrane protein	280	F1814_22	1	-1.5	1.6	-2.3	-2.6
BstC33-Mse34	CBL1 gene	70	ATJ001148	1	-1.2	-1.2	-2.6	-3.9
BstT13-Mse2	no hits	80		1	-1.0	-1.0	-4.0	-3.6
BstT33-Mse2	unknown protein AAD21709	120	AC007048	1	-1.7	-1.2	-2.1	-4.6
BstC33-Mse33	squalene epoxidase-like protein	140	CAB80441	1	1.1	1.3	-2.0	-2.0
BstC33-Mse3	unknown protein	240	AA404888	1	-1.4	-0.9	-2.5	-3.7
BstT23-Mse32	apyrase	320	At5g18280	1	-1.8	1.0	-2.3	-2.5
BctC33-Mse3	non-LTR retroelement reverse transcriptase-like-protein	180	MWJ3_8	1	1.0	-1.1	-2.6	-2.5
BstC12-Mse2	3' end of putative auxin-induced protein CAB80549	90	AL161694	1	-1.3	-1.4	-1.8	-4.5
BstC13-Mse1	putative auxin-induced protein	100	F20H23_14	1	1.3	1.4	-1.1	-2.1
BstC33-Mse3	endosomal-like protein	250	BAB10022	1	1.9	1.2	1.0	-2.1
BstC13-Mse33	Lhcb2-protein	100	AF134125	1	-0.9	-1.0	-1.5	-2.5
BstC33-Mse1	predicted protein	210	F6H11_80	1	-1.2	1.3	-1.6	-2.5
BstT13-Mse1	unknown protein	280	AY075646	1	1.5	1.3	-1.6	-2.0
BstC33-Mse3	no sequence	220		1	-1.3	-1.1	-1.8	-2.6
BstT23-Mse3	nucleotide sugar epimerase-like protein AT3g23820	110	F14O13_1	1	1.1	-1.2	-1.6	-3.1
BstC33-Mse34	GTPase ATRAB8 mRNA	200	U82434	1	2.2	1.4	1.1	0.8
BstT13-Mse2	copper transporter protein	90	At5g59030	1	1.3	2.2	1.0	-1.8
BstT23-Mse4	no hits found	150		1	1.3	2.2	-1.7	1.3
BstC33-Mse33	heat shock potein HSP17,4	200	X17293	1	2.0	4.3	-1.7	-1.7
BstC12-Mse43	intergenic region	130	AC009991	1	2.5	2.0	-1.2	-1.8
BstC13-Mse34	putative cation transport protein	250	AC007231	1	4.4	3.1	1.5	1.3
BstT23-Mse33	putative protein CAB38824	260	AL035679	1	4.4	2.9	0.9	1.8
BstC13-Mse3	At2g46600-mRNA (putative caltractin)	150	F13A10_13	1	4.2	3.0	1.0	1.3
BstC24-Mse3	ASF/SF2 homolog (SF2) gene	100	AF001035	1	2.1	2.6	1.1	1.5
BstT23-Mse3	putative fibrillin AT4g04020 AAC28198	85	T24H24_16	1	2.0	3.0	1.6	1.9
BstT21-Mse12	feebly-like protein	80	AC010870	1	4.3	3.1	1.8	1.1

pimer combination	gene or protein	length(bp)	accession	Col-0 control	30 min. 100ppb	30min. 10ppm	3hr 100ppb	3hr 10ppm
BstT13-Mse2	part of unknown protein AAD4197	140	AC006438	1	1.5	2.2	2.9	6.5
BstC13-Mse3	gene for 6-4 photolyase	70	AB017331	1	2.2	2.0	2.5	4.3
BstC32-Mse1	putative auxin-induced protein AAB82641	150	AC002387	1	2.3	1.6	2.4	2.2
BstT21-Mse34	putative protein AT5g06230	140	MBL20_11	1	2.1	1.8	3.4	2.9
Bst44-Mse2	unknown protein AAD10652	240	AC005223	1	3.1	3.1	1.9	3.2
BstC32-Mse1	putative WRKY-type DNA-binding protein At2g30250	320	T9D9_6	1	1.0	0.6	2.5	1.0
BstT23-Mse33	similar to hypothetical protein	150	T23K23_25	1	1.7	1.4	3.3	1.9
BstC24-Mse3	putative Eukaryotic translation initiation factor 5 protein	300	AY064061	1	1.0	0.5	2.1	0.8
BstC44-Mse21	putative glutathione S-transferase	410	AF387004	1	1.4	0.8	3.6	1.5
BstC13-Mse1	no hits found	90		1	1.1	1.7	3.6	1.5
BstT23-Mse43	AB08D09 cDNA clone	170	BE039006	1	1.3	1.0	2.2	1.8
BstT33-Mse2	plasma membrane intrinsic protein 1C	170	X75882	1	1.0	1.4	2.1	1.7
BstC32-Mse4	putative protein NM_120705 AT5g06230	120	AF36159	1	1.2	0.7	4.1	1.5
BstC12-Mse2	SAG20	355	AF053064	1	1.0	1.1	7.7	6.0
BctC12-Mse23	putative Ras family protein-small GTP-bindingprotein(ara-2)	90	AF332428	1	1.0	1.2	2.1	2.3
BstT12-Mse2	unknown protein AAG50811	80	AC079281	1	1.5	0.9	3.0	4.9
BstC12-Mse14	din4-gene	155	AF145452	1	0.9	0.9	2.9	8.8
BstC12-Mse21	sen1-gene	95	ATU26945	1	1.7	1.3	17.4	26.5
BstC13-Mse1	intergenic region	180	AC009322	1	1.0	1.1	4.6	4.7
BstC13-Mse1	intergenic region	185	AY039872	1	1.1	1.2	2.5	2.8
BstT13-Mse2	ERD15 protein	410	D30719	1	1.3	1.0	3.1	2.0
BstC21-Mse1	SAG21	155	AF053065	1	1.7	1.6	3.5	4.7
BstT21-Mse1	ubiquitin-conjugating enzyme 17 (UBC17) mRNA	70	AF028340	1	1.7	1.5	4.5	7.5
BstC12-Mse11	5' part of translation initiation factor-like protein BAB08773	200	AB005232	1	1.4	0.9	2.0	2.1
BstC21-Mse2	intergenic region	70	AF028340	1	1.0	1.1	3.2	6.8
BstT23-Mse2	unknown protein:AF339730	100	AF339730	1	1.8	1.4	6.6	5.8
BstT23-Mse2	unknown protein	200	AF339730	1	1.8	1.1	2.8	3.8
BstC11-Mse1	similarity to receptor-like serine/threonine kinase T3F20_25	100	AAF78446	1	1.0	1.7	3.0	3.0
BstT21-Mse2	mRNA for ferritin	170	X94248	1	1.8	1.3	2.4	3.5
BstT14-Mse2	unknown protein (At5g4510) mRNA	260	AY063839	1	1.1	1.0	2.4	3.3
BstC23-Mse2	contains similarity to helix-loop-helix DNA binding protein	120	AB013388	1	1.5	0.8	3.3	2.9
BstC14-Mse2	putative tropinone reductase	190	AC004561	1	1.5	0.9	2.9	3.1
BstC12-Mse3	ERD1 protein	230	D17582	1	1.1	1.5	2.6	2.7
BstT12-Mse3	thioglucosidase 3D precursor mRNA pyk10-gene for thioglucoside beta-glucosidase	90	AF386967	1	1.2	1.4	2.7	2.0
BstC44-Mse1	putative protein CAB45798	170	ATT12613	1	1.2	0.9	4.5	2.5
BstT33-Mse13	putative NADP-dependent oxidoreductase/zeta-crystallin-like protein	100	Z49268	1	1.2	0.9	2.6	2.2
BstC33-Mse12	putative protein CAB82975 T7H20_70	200	ATT7H20	1	1.0	1.0	2.7	5.6
BstT22-Mse3	putative protein AAF79235	420	AC006917	1	1.2	1.2	2.3	2.5
BstT33-Mse1	Mus Musculus/Homo Sapiens hit	220		1	1.1	0.6	2.0	2.3
BstC32-Mse1	putative protein CAB41160 (contains ATP/GTP-binding site motif A)	220	T29H11_100	1	1.3	0.9	2.3	2.0
BstT23-Mse31	Arabidopsis thaliana ATP-citrate lyase subunit A mRNA	210	AY056593	1	1.6	0.8	4.0	2.2
BstT44-Mse2	sen1	220	ATU26945	1	1.0	1.1	16.1	12.5
BstT22-Mse32	unknown protein BAB08813	80	MLE2_11	1	1.4	1.6	2.2	2.5
BstC44-Mse21	putative topoisomerase	220	AY064061	1	1.2	1.5	2.4	3.3
BstC14-Mse2	unknown protein	150	F14J22	1	1.3	1.6	2.7	4.1
BstC14-Mse24	ubiquitin-conjugating enzyme 5 (UBC5) mRNA	130	zl19356	1	1.2	1.2	2.5	2.3
BstC44-Mse2	unknown protein	110	AF349515	1	1.0	0.8	4.5	3.4
BstC33-Mse2	no sequence	120		1	1.7	1.8	9.4	4.6
BstC44-Mse24	unknown protein AAF0411 - T22K18_4	390	AC010927	1	1.6	1.6	5.7	3.6
BstT23-Mse41	unknown protein AAC63658 At2g23840	320	T29E15_4	1	1.9	1.4	3.3	3.6
BstC23-Mse3	hypothetical protein AAC16455	200	AC003058	1	1.1	1.7	3.4	5.0
BstT23-Mse3	unknown protein- At3g14990	100	K15M2_13	1	1.0	0.9	2.4	2.5
BstC33-Mse34	no sequence	120		1	1.8	1.0	2.8	2.3
BstT44-Mse1	dormancy-associated protein (DRM1)	100	AF053747	1	1.1	1.0	2.3	3.1
BstC12-Mse2	between K2A18_15 and KA18_16	110	AB011474	1	-1.4	-1.6	2.2	2.0
BstC33-Mse33	Lycopersicon esculentum cotyledon: cDNA clone fragment 180 similar to putative cytochrome P450 from A.th (AC002391)	160	AF211810	1	-1.9	-1.5	2.0	2.1
BstC13-Mse2	no sequence	420		1	-1.3	-1.3	2.9	4.1
BstT23-Mse4	BAB09228-protein	290	MDF20_5	1	-1.4	-1.0	2.6	4.5
BstT23-Mse42	pyruvate decarboxylase BAB08775	310	MBG8_23	1	1.6	-1.7	2.8	2.1
BstC33-Mse3	putative beta-galactosidase (BGAL1-gene)	100	ATH270297	1	1.4	-1.4	3.3	2.6
BstT23-Mse3	unknown protein AT3g14990	90	K15M2_13	1	-1.7	-1.7	1.8	2.3
BstC12-Mse14	AT5g1616501/MTG13_10 mRNA	80	AF385723	1	0.9	0.8	1.6	2.7
BstC12-Mse22	unknown protein:AAD21434	380	AC006921	1	1.9	1.3	1.3	3.0
BstT33-Mse3	unknown protein	120	AY034967	1	1.0	0.7	1.4	5.4

pimer combination	gene or protein	length(bp)	accession	Col-0 control	30 min. 100ppb	30min. 10ppm	3h 100ppb	3h 10ppm
BstC12-Mse2	unknown protein	120	AAF78445 AAF78446	1	1.2	0.9	1.8	2.1
BstT33-Mse11	metallothionein-like protein	340	ATU15108	1	0.8	1.0	1.1	5.8
BstT14-Mse31	unknown protein	280	AY046029	1	0.7	0.7	1.8	2.5
BstC13-Mse1	unknown protein	120	At5g44010	1	1.1	1.5	1.5	2.1
BstC12-Mse2	unknown protein T19N8_1	255	AP002057	1	1.3	1.6	1.4	2.8
BstT14-Mse22	CBL1(cystathionine beta-lyase)-gene	200	AJ001148	1	1.0	0.9	1.8	2.2
BstT23-Mse42	putative serine/threonine protein phosphatase type one	280	F18C1_15	1	-1.1	-2.1	1.9	2.5
BstT13-Mse32	contains similarity to transfactor gene_1d:MBK21.11,protein: BAB02417	230	AB024033	1	-1.2	-2.2	1.2	1.4
BstT14-Mse2	unknown protein AAC27848	430	AC004218	1	-1.8	-2.4	11.1	8.1
BstT14-Mse2	putative protein CAB79773-AT4g30550	150	F17I23_110	1	-1.1	-2.2	2.4	1.9
BstC44-Mse22	unknown protein K15M2_13 AT3g14990	200	AY039574	1	-1.9	-2.1	5.4	3.5
BstC33-Mse14	MPK5	120	D21841	1	-2.2	-2.6	2.7	3.7
BstT22-Mse34	no sequence	520	AF178073	1	-5.3	-2.8	1.7	2.9
BstT33-Mse11	no Arabidopsi hits	110		1	-2.9	-1.5	2.2	2.5
BstC33-Mse33	At1g33140/T9L6_10 mRNA	410	AY039593	1	1.8	2.0	-3.9	-2.7
BstC33-Mse3	no sequence	80		1	1.1	2.3	-3.3	-1.2

2.3.1.2 Focused cDNA-AFLP experiment

To perform a more detailed analysis of the very early responses to ethylene, we set up a kinetic analysis over 6 time points. Plants were treated with ethylene for 10 min., 20 min., 30 min., 1 h, 2 h, and 6 h. The samples harvested after 6 hours of treatment allowed us to investigate whether mRNA levels of the early induced genes remained constant during longer periods of ethylene exposure. In order to confirm that the genes are modulated by ethylene, the ethylene-insensitive mutant *ein2-1* and the constitutive ethylene-response mutant *ctr1-1* were included. The *ein2-1* mutant was chosen because *EIN2* is the only known gene for which a loss-of-function mutation leads to complete ethylene insensitivity (Roman *et al.*, 1995). Consequently it could be expected that ethylene-regulated genes were unaffected by the treatment in *ein2-1* and would display constitutive expression in *ctr1-1*. In order to reveal genes that respond differentially to low and high concentrations of ethylene, treatments were done using two different concentrations of ethylene, 100ppb and 10ppm. A substantial number of genes are known to be regulated by the circadian clock (Harmer *et al.*, 2000). Because the first (10 min.) and the last (6h) time points were 5h 50 minutes apart, untreated controls at both time points were included for wild type and mutants. Genes controlled by the circadian clock or mechanical stress during the treatment with ethylene, should be regulated in the same manner in air or ethylene-treated wild type plants and in the mutants. Figure1 (b) shows *CAB* (chlorophyll a/b binding protein) gene expression, which is known to be negatively regulated by the circadian clock (Millar and Kay, 1996). *CAB* was amplified by one of the chosen primer combinations and displayed an expression pattern presumably regulated by the circadian clock (fig. 2). In this way, most genes that are under circadian control could be eliminated.

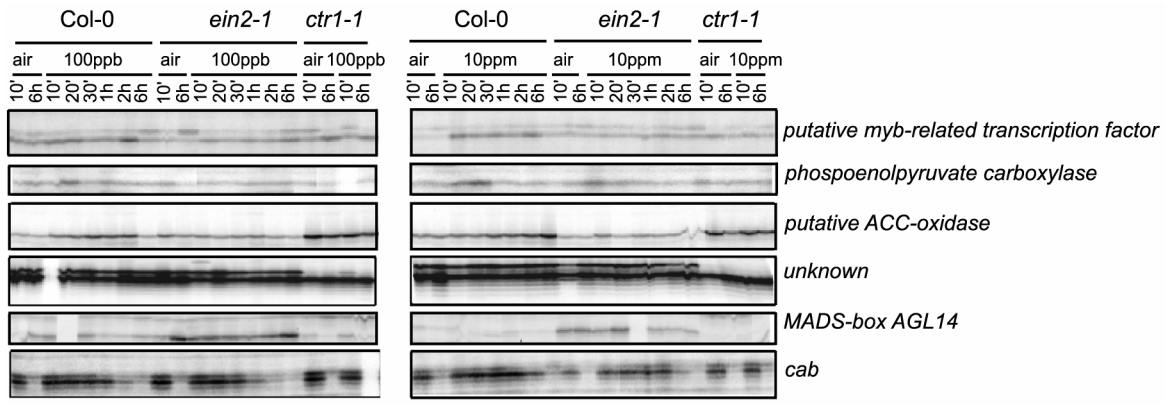


Figure 2: **Different reproducible expression patterns of transcripts by using the cDNA-AFLP transcript profiling approach**. Left panel represent the expression patterns after treatment with 100ppb ethylene, right panel represents that after treatment with 10ppm ethylene. Col-0 and *ein2-1* samples were treated with ethylene for 10, 20, and 30 minutes, 1, 2, and 6 hours (': minutes; h: hours in figure). Control samples were treated with air for 10 minutes and 6 hours. The *ctr1-1* samples were treated with ethylene for 10 minutes and 6 hours. Labeling was done with ^{33}P and separation was performed on a 5% polyacrylamide gel. Distinct expression patterns could be observed, in order of appearance: transient up-regulation of mRNA level after short treatment with ethylene; peak in expression level during the 10 to 20 minutes of treatment; ethylene-regulated expression pattern with a stimulatory effect of ethylene in time; very low in *ctr1-1* compared to the wild type and *ein2-1*; elevated mRNA level in the *ein2-1* mutant; expression pattern regulated by the circadian clock.

In this analysis 10 primer combinations with selectivity +2/+1 and 7 primer combinations with selectivity +2/+2 were used, representing an estimated 5% of the *Arabidopsis* transcriptome. Figure 2 shows distinct expression patterns observed on the AFLP gels. A total of about 1200 transcripts were monitored. After normalization of the AFLP-QuantarPro expression data and a statistical analysis to select differentially expressed genes, 47 genes displayed a significantly (with a coefficient of variation > 0.5) altered expression pattern upon ethylene exposure. Interestingly, 10 genes out of the 47 genes, revealed a temporal increase in mRNA levels shortly (10min.) after the beginning of the treatment. Some of these transcript tags reached their maximum levels after 10 to 20 minutes (example in fig. 2: phosphoenolpyruvate carboxylase); while others displayed their highest mRNA levels during a longer period of treatment (example in fig. 2: putative myb-related transcription factor). Furthermore, some transcripts displayed a pattern with a stimulatory effect of ethylene in time in Col-0, low mRNA levels in *ein2-1*, and high levels in *ctr1-1* (example in fig. 2: putative ACC-oxidase). In addition, for 3 tags the level of expression was equal in Col-0 and *ein2-1,* but very low or zero in the *ctr1-1* mutant (example in fig. 2: unknown protein). Finally, patterns were observed with an elevated expression restricted to the *ein2-1* mutant (example in fig. 2: MADS-box gene AGL14). A comparable pattern was observed for 6 tags. Differentially expressed transcript tags were excised from the gels, reamplified with the selective primers, and sequenced. Significant

homology with *Arabidopsis* genes of known or suggested function was found for 37 of the transcript fragments, whereas 8 tags were homologous to genes of unknown function. For 2 of the tags, no reliable sequence could be obtained (displayed in the figures as unidentified tags).

2.3.2 Identification and characterization of ethylene-modulated genes by clustering

In figure 3, the hierarchical average linkage clustering of the 47 differentially expressed genes for both treatments is presented (Eisen *et al.*, 1998). Based on this clustering, we were able to distinguish 4 main expression profiles, designated A, B, C, and D. In the first group (Fig. 3 (A)) expression was very rapidly modulated and transient, with maximum mRNA levels after 10 and 20 minutes of treatment. The common feature for a second group (Fig 3 (B)) is the high expression in *ctr1-1*. Genes that are up-regulated after prolonged exposure to ethylene are part of this group. The expected ethylene-insensitive pattern for these genes in the *ein2-1* mutant is observed. A third profile (Fig. 3 (C)) revealed an early temporal increase in mRNA levels during the treatment. This cluster can be divided in 2 sub-clusters, according to their expression profiles. In the first subcluster (Fig. 3 (C-1)) the peak of expression occurred after 1h-2h of treatment. In the second subcluster (Fig. 3 (C-2)), up-regulation by ethylene was seen after 10 minutes of treatment and remained high until 2h. Finally, a fourth expression profile is seen in cluster D, with a low expression in *ctr1-1*. Here again, it was possible to classify the expression profiles in two subclusters. In the first subcluster (Fig. 3 (D1)), mRNA levels were specifically low in the *ctr1-1* mutant, whereas comparable mRNA levels were seen in the wild type and the *ein2-1* mutant. This was not the case for the second subcluster (Fig. 3 (D2)), where a higher expression level could be observed in the *ein2-1* mutant, indicating that these genes are possibly down-regulated by ethylene.

In parallel to the Eisen clustering, we also performed a second clustering using the adaptive quality-based method (De Smet *et al.*, 2002). Six expression profiles were defined similar to the result obtained by the Eisen clustering (Fig. 4). All profiles in fig. 4 represent the normalized expression profiles of the genes treated with 10ppm. The level of expression for transcript tags in cluster A (Fig. 4(A)) increased rapidly (within 10 min.), while for the *ein2-1* mutant and the *ctr1-1* mutant no significant change in mRNA level was observed. The genes belonging to cluster B (Fig. 4(B)) showed an induction of gene expression upon exposure to ethylene, increasing with time. Additional proof for true ethylene-regulation is given by the ratio of expression in *ein2-1* profiles over the 6h time

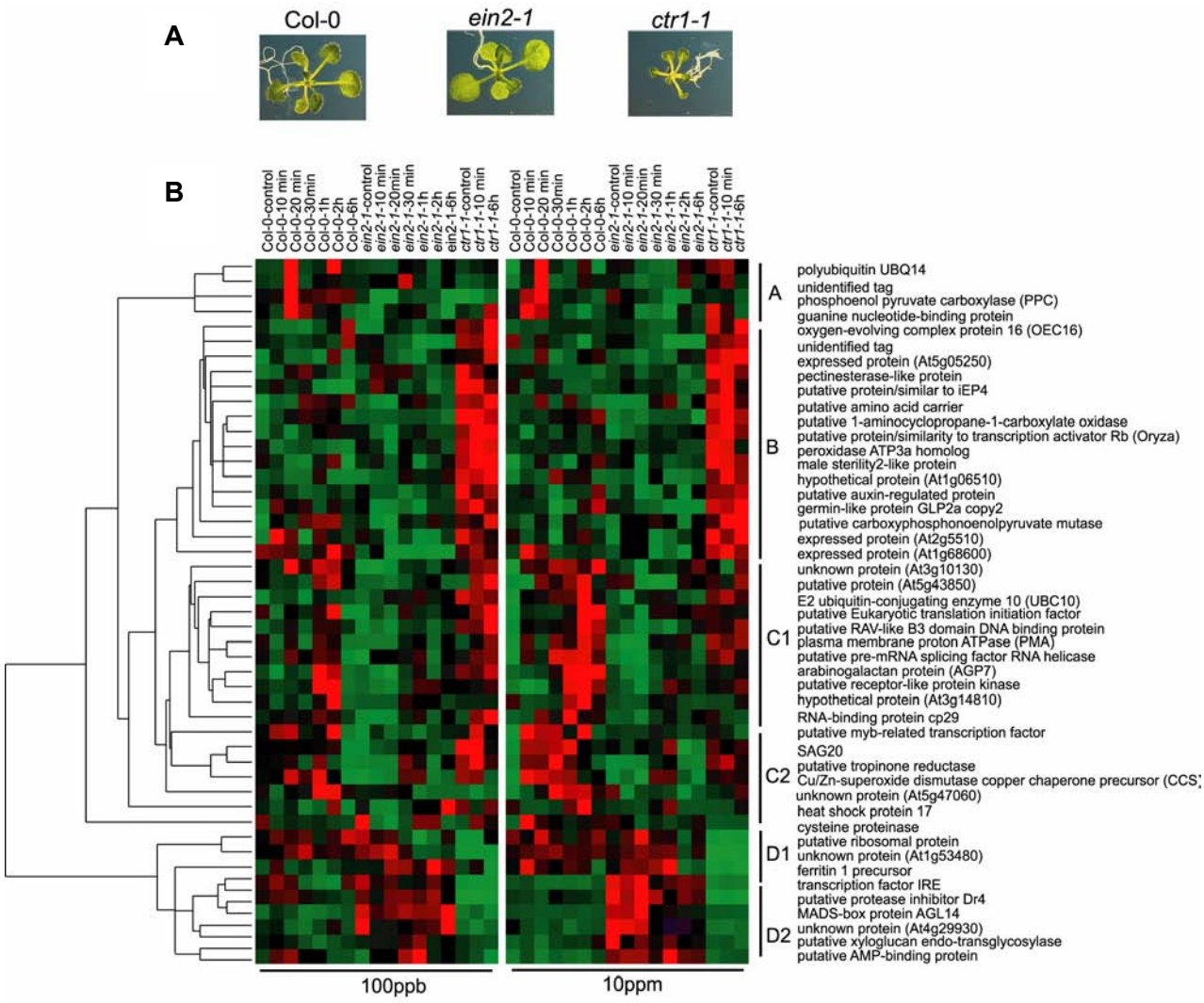
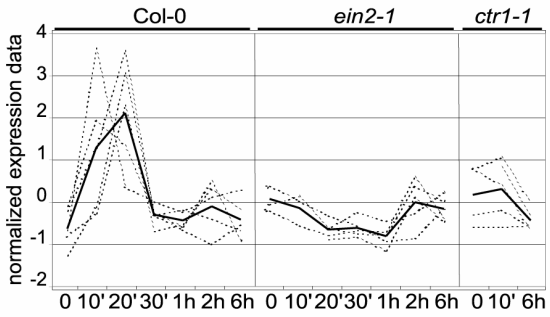


Figure 3: Hierarchical clustering of the isolated ethylene-regulated transcript tags. A. Phenotype of wild type and ethylene mutants *ein2-1* and *ctr1-1* at the developmental stage used in this TP-analysis B. Eisen clustering: each column represents a time point of sampling during the treatments for wild type, *ein2-1* and *ctr1-1* and each row represents the expression profile of a tag shown over the 7 time points for Col-0 and *ein2-1.* For the *ctr1-1* samples the results are displayed for the 3 time points. Red and green reflect transcriptional activation and repression respectively. The main expression patterns are divided in 4 groups. The significant homology of the tags to known and unknown sequences are indicated on the right. Two tags (unidentified tags in fig.) for which no reliable sequences could be retrieved are included. On the left side, the data from the 100ppb treatment are presented, on the right the data from the 10ppm treatment.

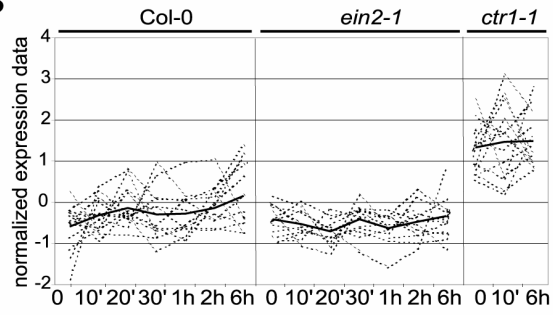
course showing no significant difference. On the contrary, for *ctr1-1*, expression was high at all time points. The initial expression profile of cluster C (Fig. 4(C)) was similar to that of cluster B, but expression levels decreased at the last time point of ethylene treatment (6h). For about half of the genes in this cluster the *ctr1-1* expression levels were elevated whilst for the other half of the genes no significantly higher expression in the *ctr1-1* mutant was observed. The genes in cluster D (Fig. 4 (D)) showed a similar expression profile as seen for the transcripts in cluster A. The difference between both clusters is that the transcript tags in cluster D remained up-regulated over a longer period (until 2h after the beginning

A



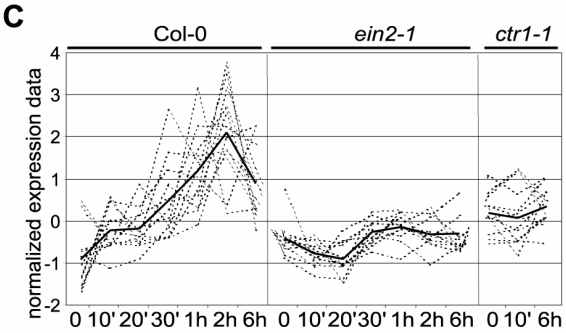
Cluster A	Number of genes: 5
putative guanine nucleotide-binding protein (*)	At1g48630
cysteine proteinase RD21A (*)	At1g47128
polyubiquitin (UBQ14) (*)	At4g02890
unidentified tag (*)	
phosphoenolpyruvate carboxylase (PPC) (*)	At3g14940

B



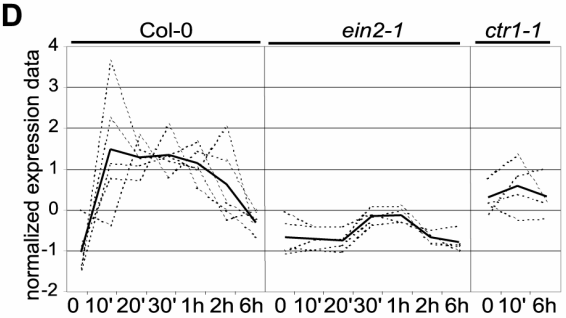
Cluster B	Number of genes: 15
putative carboxyphosphonoenolpyruvate mutase (*)	At2g43180
male sterility2-like protein (*)	At5g22500
oxygen-evolving complex protein 16 (OEC16) (**)	At4g21280
unknown protein (*)	At1g06510
putative auxin-regulated protein (**)	At2g45210
expressed protein (*)	At5g05250
putative 1-aminocyclopropane-1-carboxylate oxidase (**)	At1g62380
putative amino acid carrier (*)	At1g77380
bHLH protein family protein (*)	At3g50330
peroxidase ATP3a homolog (*)	At5g64110
germin-like protein (GLP2a) copy2 (*)	At5g39190
pectinesterase like protein (**)	At3g49220
similar to iEP4	At1g67830
unidentified tag (**)	
unknown protein	At2g25510

C

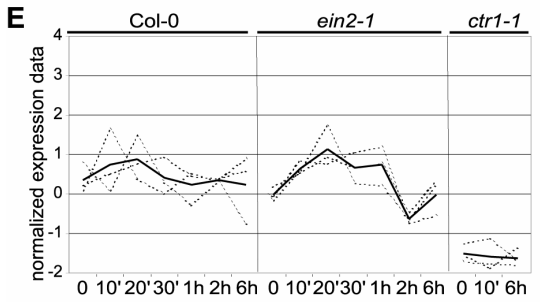


Cluster C	Number of genes: 12
unknown protein (*)	At3g10130
RNA-binding protein cp29 (*)	At3g53460
heat shock protein 17 (*)	At3g46230
arabinogalactan protein (AGP7) (*)	At5g65390
receptor-like protein kinase, putative (*)	At1g11330
plasma membrane proton ATPase (PMA) (*)	At2g18960
E2. ubiquitin-conjugating enzyme 10 (UBC10) (*)	At5g53300
putative pre-mRNA splicing factor RNA helicase (*)	At2g35340
unknown protein	At3g14810
putative RAV-like B3 domain DNA binding protein (*)	At2g46870
putative Eukaryotic translation initiation factor (*)	At1g36730
unknown protein	At5g43850

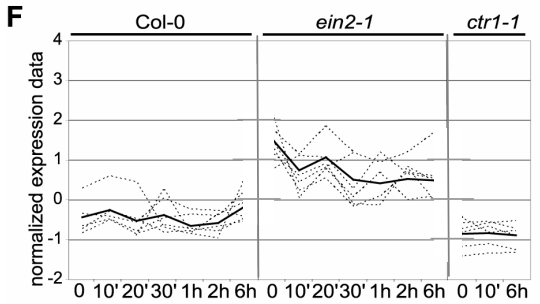
D



Cluster D	Number of genes: 6
unknown protein (**)	At1g68600
SAG20	AF053064
Cu/Zn-superoxide dimutase copper chaperone precursor(*)	At1g12520
putative tropinone reductase (*)	At2g29310
putative myb-related transcription factor (*)	At1g22640
unknown protein (**)	At5g47060



Cluster E	Number of genes: 3
putative ribosomal protein	At3g16080
ferritin1 precursor	At5g01600
unknown protein	At1g53480



Cluster F	Number of genes: 6
MADS-box protein AGL14	At4g22950
putative bHLH transcription factor	At4g29930
putative AMP-binding protein 2 (*)	AF503761
putative xyloglucan-endotransglycosylase (*)	At2g18800
transcription factor IRE (*)	At1g48490
putative protease inhibitor Dr4 (*)	At1g73330

Figure 4: **Gene expression profiles obtained by adaptive quality-based clustering (AQBC)**. The normalized data from the treatment with 10ppm are used for this clustering. Dashed lines represent the normalized expression profiles of the genes The full line graphs represent the mean expression profile of all transcripts belonging to the respective cluster Values on the y axis are the normalized expression data A. Cluster of transcripts with maximal mRNA level after 10-20 minutes of treatment B.Cluster of transcripts displaying an ethylene-induced expression profile C. Cluster of transcripts with peak expression after 1-2h of treatment D. Cluster of transcripts displaying a temporal increase in mRNA level after 10 minutes of treatment up to 2 hours of treatment E. Cluster of transcripts with lower expression level in the *ctr1-1* mutant F. Cluster of transcripts with higher expression level in the *ein2-1* mutant. For each expression profile, the genes are listed with their respective chromosome locus numbers and function. The total number of genes belonging to each cluster is indicated.
Thirty transcripts with a higher mRNA level in the presence of 10ppm compared to 100ppb are indicated by (*), seven transcripts with a higher mRNA level in the presence of the 100ppb compared to 10ppm are indicated by (**).

of the treatment), while the transcript tags in cluster A are specifically up-regulated over a short time period (until 20 minutes)). Contrary to the previous clusters, cluster E and F display transcripts that are down-regulated, either these transcripts showed a low mRNA level in the *ctr1-1* mutant (cluster E), either these transcripts showed a higher level of expression in the *ein2-1* mutant (cluster F). For all the clusters, the transcript tags encode proteins playing diverse functions in plants.

This analysis also revealed concentration dependence of ethylene response. Hua and Meyerowitz (1998) suggested that one function of the ethylene receptors might be to enable the plants to sense ethylene over a wide range of concentrations (Hua and Meyerowitz, 1998). Each of these receptors may have a different affinity for the hormone. We were interested to investigate whether differences occur on the transcriptional level in response to a low and a higher concentration of ethylene. By comparing the results of both

treatments, the isolated genes could be divided in three groups. One group showed a quantitatively similar expression pattern in both treatments. The second and largest group (containing 30 genes) showed a stronger response to the 10ppm treatment of ethylene (see Fig. 4. indicated by (*)). Interestingly, for a third group (7 genes) the response was higher to the treatment with the lower concentration of ethylene (see Fig. 4. indicated by (**)).

2.3.3 Ethylene regulation of genes involved in the ubiquitin-mediated degradation pathway

Recent studies have connected individual components of the ubiquitin/26S proteasome pathway to almost all aspects of plant development, including hormone signalling (Kepinski and Leyser, 2002; Koyama *et al*., 2003; McGinnis *et al.,* 2003; Smalle *et al*., 2002; Smalle *et al*., 2003, Guo and Ecker, 2003; Potuschak et al., 2003). Two genes involved in the ubiquitin degradation pathway in plants, *UBC10* and *UBQ14*, were early induced by ethylene. This observation led to the hypothesis that ubiquitin-mediated degradation is possibly involved in ethylene signalling. To find evidence for this hypothesis, we investigated the response to ethylene of genes belonging to different families of components involved in ubiquitin-mediated proteolysis (Table 2) using RT-PCR.

Table 2: Primer sequences used in RT-PCR analysis of proteins involved in the ubiquitin-mediated degradation pathway

Gene	Accession number	Forward primer	Reverse primer	Ethylene response
UBC3	At5g62540	5'-ATGTGAGAACTGCAGCAACG-3'	5'-AGGGCGGATACTGAGGAAAT-3'	no
UBC4	At5g41430	5'-TCCTTGAACCAGCTTTGG-3'	5'-TAAACAGGTGAACAAAGC-3'	no
UBC5	At1g63800	5'-AGGGCGGATACTGAGGAAAT-3'	5'-GTTTACCGGCGATTGCAACA-3'	no
UBC8	At5g41700	5'-TGGTACCAGAGATTGCACAC-3'	5'-AACAGGTGCTGAAGCATACG-3'	no
UBC13	At3g46460	5'-CATCCTCCTGGTGATGATCC-3'	5'-TTTCTGACACAACGGCTCAC-3'	no
putative UBC	At5g50430	5'-AGCTGGAATCCAATGTGGTC-3'	5'-GTTGCTGGCTGTACTTCTCG-3'	no
putative UBC	At2g16740	5'-GATCCAAACCCTGACGAC-3'	5'-TACTTCTGGGTCCAGCTTCG-3'	no
putative UBC	At1g50490	5'-GACTGTTGACAGCCAATCTG-3'	5'-GGAGGCTTGAAAGGATAGTC-3'	no
putative UBC	At2g16920	5'-CCGGGTATGATAAGCAGGTC-3'	5'-TCCTTTCATGTAGGCGTCAC-3'	yes
putative UBC	At1g53020	5'-AGGGAACTCCTTACCATGACG-3'	5'-CGACTCATTTGGGAGCCATT-3'	no
putative UBC	At2g33770	5'-ACGGGTCTGGTGAAGCTCTA-3'	5'-TGAACAAGGACCTCAGAGTCAC-3'	no
UBQ5-like	At1g23410	5'-CAGCAACGGTTGATCTTCG-3'	5'-TCTTCTTCGCACCTCCTCTC-3'	no
UBQ10	At4g05320	5'-GAGGTATTCCTCCGGACCAG-3'	5'-TAGAAACCACCACGAAGACG-3'	no
UBP4	At2g22310	5'-CACAAGCCACAAAAGCAGAC-3'	5'-TCAAGGCTCAGGTCTAGGAAAG-3'	yes
UBP23	At5g57990	5'-GGAGGATTCAAAGGCTGCAT-3'	5'-AGGTCCAAGGGTAGGCAACT-3'	yes
UBP24	At4g30890	5'-CACAGATGCTCGACGAAAGA-3'	5'-ACTCAGAAGACCGGACACAAG-3'	no
UBP27	At4g39370	5'-TAGAGGATGCGAGAGGTTCG-3'	5'-AGAAGGGCTTCTGCTGCAT-3'	yes
RUB1	At1g31340	5'-GGGCATACCACCTGATCAAC-3'	5'-CGATCAATAGTGTCGGTTGG-3'	no
COP10	At3g13550	5'-TAACTAACCAAAGAACTAGG-3'	5'-GACTGCGTAGTGATCT-3'	no
RCE1	At4g36800	5'-CCCTTGAACCATGATGCTG-3'	5'-CAAACGAGGGTCCTTGAGAA-3'	no
EBF1	At2g25490	5'-TCCTATCAGTTGCAGGTTGC-3'	5'-CCCAATAGAGTGGAACCCAAC-3'	no
putative UCH	At3g11910	5'-GTCCCTGATGAGGACTTTGC-3'	5'-TCGTATGCGTGTCGGTTCT-3'	no

Abbreviations used in table 1: UBC: ubiquitin-conjugating enzyme, COP: constitutive morphogenic, RCE: Rub1-conjugating enzyme, UBQ: ubiquitin, RUB: related to ubiquitin, UBP: ubiquitin-specific protease, EBF: EIN3-binding F-box protein, UCH: ubiquitin carboxyl-terminal hydrolase.

PCR-amplifications were done on the preamplification products of wild type, *ein2-1* and *ctr1-1* treated with 10ppm and 100ppb of ethylene respectively (see experimental procedures). In total 22 transcripts were examined and those for which an ethylene-regulated pattern was observed are shown in Fig. 5. Three transcripts belonging to the family of ubiquitin specific proteases (UBPs) exhibited ethylene-inducible expression. *UBP4* displayed a higher, albeit transient expression after 10 and 30 minutes of treatment in the wild type. UBP23 was up-regulated by ethylene in time. The mRNA level in the *ctr1-1* mutant was higher compared to the baseline expression level in the *ein2-1* mutant. For UBP27 higher expression was observed in the presence of ethylene, while expression remained unaffected in *ein2-1* and expression was elevated in *ctr1-1*. Furthermore, one gene encoding a putative ubiquitin conjugating enzyme (UBC), possibly functioning as an E2 enzyme in the process of ubiquitylation, was found to be ethylene-regulated. This putative UBC (At2g16920) exhibited a transient peak in expression levels within 20

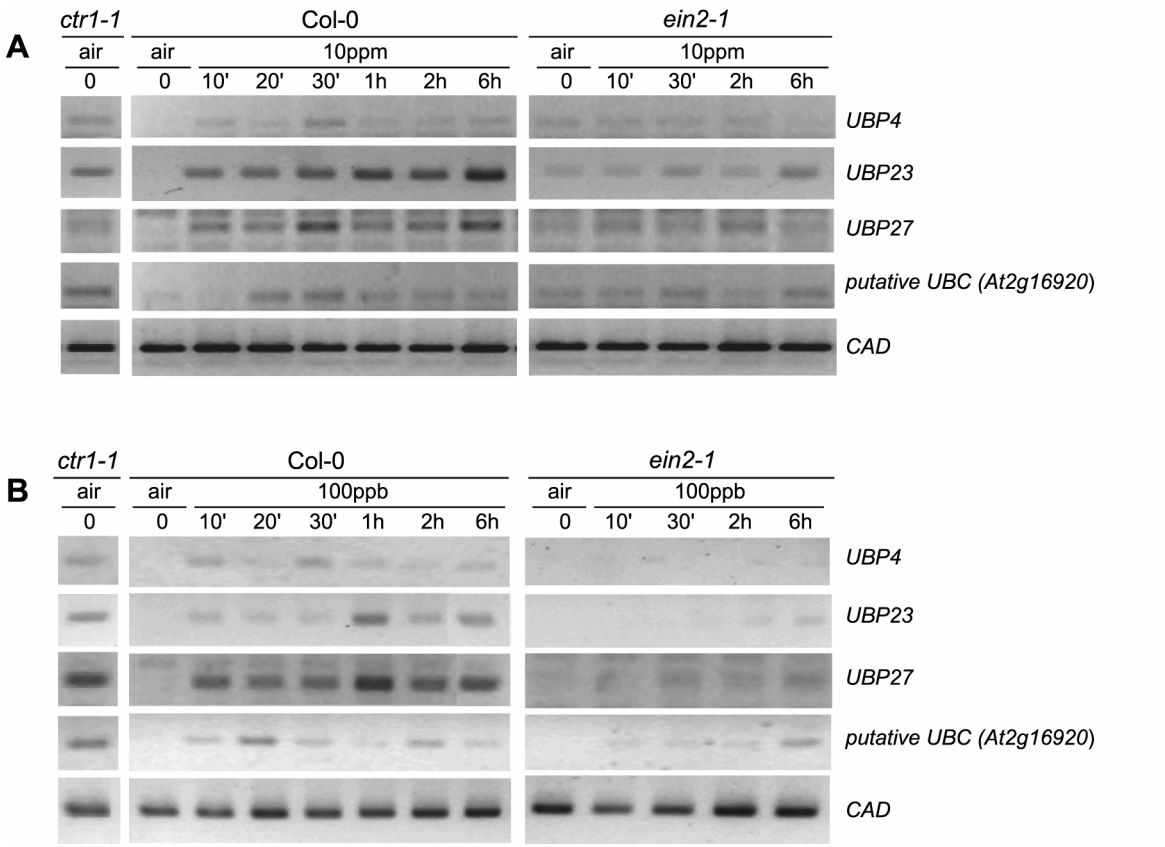


Figure 5: Analysis of the steady state messenger levels of genes encoding components of the ubiquitin-mediated protein degradation pathway after treatment with 10ppm and 100ppb of ethylene. Amplifications were performed on the preamplification products for cDNA-AFLP analysis. Specific oligonucleotide primers were used to detect the transcript levels of the genes listed in table 2. A. An EtBr stained gel of the amplification products for UBP4 (20 cycles), UBP23 (20 cycles), UBP27 (27 cycles), and a putative UBC At2g16920 (20 cycles) from the 10ppm treatment is shown. CAD was used as a control (30 cycles) (Moshkov *et al*., 2003) B. An EtBr stained gel of the amplification products for UBP4 (20 cycles), UBP23 (25 cycles), UBP27 (27 cycles), and a putative UBC At2g16920 (25 cycles) from the 100ppb treatment is shown. CAD was used as a control (30 cycles).

minutes of exposure to the hormone. Moreover, this gene displayed a higher mRNA level in the *ctr1-1* mutant, comparable to that seen for the peaks of expression in the wild type.

2.4 DISCUSSION

The aim of this study was to identify early ethylene responsive genes in *Arabidopsis* plants. To this end, a time-course cDNA-transcript profiling experiment was performed to screen for novel genes in the transcriptional cascade in response to ethylene. Database searches revealed that 77 % of the 47 isolated tags were highly homologous to genes with known function, and 23 % of the tags matched a cDNA or genomic sequence with a yet unknown function. An overview of the isolated genes and their known or putative function is listed in table 3. Previous research in tomato revealed that ethylene-dependent changes in mRNA accumulation occur very rapidly (Zegzouti *et al.*, 1999). Also in this study, we identified transcripts (Fig. 4, cluster A, C and D) displaying a temporal increase in mRNA levels during the earliest periods of ethylene treatment. These genes encode proteins functioning in distinct metabolic and regulatory processes, supporting the pleiotropic role of the hormone. Furthermore, treatment with ethylene for a long period results in a dramatic change in phenotype confirming that many processes are affected in the plant.

2.4.1 Early ethylene response genes playing a role in protein degradation

One striking result from this study, is the link between ethylene and protein degradation. Ubiquitin-mediated proteolysis has emerged as being fundamentally important in many aspects of development such as hormone signalling, light perception, and circadian rhythm, as well as in plant defence signalling (Callis and Vierstra, 2000; Kepinski and Leyser, 2002; Kim and Delaney, 2002; Koyama *et al*., 2003; Liu *et al*., 2002; Xu *et al*., 2002). In this pathway, ubiquitin becomes covalently attached to cellular proteins by an ATP-dependent reaction cascade, which requires three distinct enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3). UBPs (ubiquitin-specific proteases) help regulate the ubiquitin/26S proteolytic pathway by generating free ubiquitin monomers from their initial translational products, recycling ubiquitins and/or by removing ubiquitin from specific targets and thus presumably preventing target degradation (Ning Yan *et al*., 2000). Until now, a role for protein degradation has been demonstrated in auxin, jasmonate, cytokinin, absiscic acid and GA signalling (Kepinski and Leyser, 2002; McGinnis *et al.,* 2003; Smalle *et al.,* 2002; Smalle *et al*., 2003). As for ethylene signalling, one report described the interaction of the tobacco

Table 3: Overview of isolated genes with homology to known genes

Gene and function	Cluster	Comments	References
Protein degradation:			
E2 ubiquitin-conjugating enzyme 10 (UBC10)	C	New	
polyubiquitin(UBQ14)	A	pSEN3 encodes a polyubiqitin	Park *et al*, 1998
Transcriptional and post-transcriptional regulation:			
putative myb-related transcription factor	D	New	
MADS-box gene AGL14	F	New	
putative RAV-like B3 domain DNA binding protein	C	New	
putative bHLH transcription factor	F	New	
bHLH protein family protein	B	similarity to transcription activatorRb (Oryza sativa)	
putative eukaryotic translation initiation factor 5(eIF5)	C	elongation factor EF-Ts isolated in tomato	Zegzouti *et al.*, 1999
RNA-binding protein cp29	C	New	
putative pre-mRNA spilicing factor helicase	C	strong homology to DEAH/DEAD box helicase mRNA helicase gene isolated in tomato	Zegzouti *et al.*, 1999
Involved in cell wall:			
putative xyloglucan endo-transglycosylase (XET)	F	New	
arabino galactan protein (AGP7)	C	New	
pectinesterase-like protein	B	New	
Signalling and regulatory events:			
Plasma membrane proton ATP-ase (PMA)	C	New	
putative receptor-like protein kinase	C	New	
IRE (incomplete root hair elongation)	F	New	
putative guanine nucleotide-binding protein	A	GTP-binding proteins in ethylene signalling ethylene-regulated GTP-binding proteins in *Arabidopsis*	Novikova *et al.*, 1999 Moshkov *et al.*, 2003
putative AMP-binding protein2	F	New	
ferritin1 precursor	E	New	
Involved in defence mechanisms:			
SAG 20	D	Known ethylene-regulated gene	Weaver *et al.*, 1998
Cu/Zn-superoxide copper chaperone precursor	D	New	
heat shock protein 17 (HSP17)	C	heat-shock cognate 70 gene (HSC70) isolated in tomato	Zegzouti *et al.*, 1999
cysteine proteinase RD21A	A	dehydratation-regulted gene senesence-associated gene	Koizumi *et al.*, 1993 Weaver *et al.*, 1998
germin like protein (GLP2a) copy2	B	New	
peroxidase ATP3a homolog	B	New	
Involved in hormone signalling:			
putative auxin-regulated protein	B	New	
putative 1-aminocyclopropane-1-carboxylate oxidase	B	New	
Involved in metabolic processes:			
putative carboxyphosphoenolpyruvate mutase	B	putative carboxyphosphoenolpyruvate mutase isolated in senescent carnation	Wang *et al.*, 1993
male sterility2-like protein	B	Role of ethylene in male gametogenesis	Dahnous *et al.*, 1982
putative amino acid carrier	B	New	
phosphoenolpyruvate carboxylase (PPC)	A	New	
putative tropinone reductase	D	New	
Involved in photosynthesis:			
Oxygen-evolving complex protein 16 (OEC16)	B	New	
Unclassified proteins:			
Major latex protein (MLP)-related	F	New	
putative protease inhibitor Dr4	F	New	
putative ribosomal protein	E	New	
unknown protein/similar to nodulation inception protein	F	New	

ERF3 transcription factor with NtUBC2 in a yeast two-hybrid system (Koyama *et al*., 2003). In addition, the *Arabidopsis wei1* (weak ethylene insensitive 1) mutant is described as a plant with a mutation in *TIR1*, a gene that participates in an ubiquitin-mediated degradation of general components of the auxin response (Alonso *et al.*, 2003). Direct evidence that ubiquitin-mediated protein degradation is important in ethylene signalling is given by Guo and Ecker and Potuschak et al. (2003). They demonstrated that in the absence of

ethylene, EIN3 is quickly degraded through a ubiquitin/proteasome pathway mediated by two F box proteins, EBF1 and EBF2. These results revealed that a ubiquitin/proteasome pathway negatively regulates ethylene responses by targeting EIN3 for degradation. In our transcriptome-analysis, the ubiquitin conjugating enzyme 10 (*UBC10*) (Fig. 4, cluster C), an E2 protein, was isolated as an early ethylene response gene, peaking at 1-2h of treatment. *UBQ14*, another ubiquitin related gene, was identified in the class of very early ethylene-regulated genes with maximal expression after 10'-20' of treatment (Fig. 4, cluster A). Polyubiquitin (*UBQ*) genes encode precursor proteins which need proteolytic processing by previously described UBPs to release mature ubiquitin which on its turn is covalently attached to substrate proteins usually targeting them for degradation (Bachmair *et al.*, 2001). In agreement with our results, the pSEN3 clone, first isolated as a senescence-induced gene, showed an increase upon ethylene exposure and was found to encode a polyubiquitin (Park *et al.*, 1998). The response to ethylene for a number of components involved in the process of ubiquitin-mediated proteolysis was further investigated by analyzing their steady state messenger RNA levels. Interestingly, the *EBF1* mRNA, did respond to ethylene in our RT-PCR analysis; however, this was also true for the insensitive control mutant. Similar results were observed in our microarray analysis (see Chapter 4, p85). Therefore this expression profile probably reflects a circadian regulation rather than by ethylene itself. This observation is in contrast to the described results of Guo and Ecker (2003), and Potuschak et al. (2003). One reason could be the different tissue and age of plants used in our analysis. However, a clear difference in expression between the zero time-point and 1h of ACC treatment in *ein3-1* background is also visible in the paper of Potuschak et al., corroborating our results. In contrast, three UBPs and one putative UBC exhibited an ethylene-regulated expression profile. All together, we can conclude that responses of plants to ethylene also require specific components of the ubiquitin/proteasome pathway, much comparable to what is seen in other hormone response pathways.

2.4.2 Early ethylene response genes involved in transcriptional and post-transcriptional control of gene expression

Five transcription factors were identified, each with a distinct expression profile. A putative myb-related transcription factor (Fig. 4, cluster D) and a putative RAV-like B3 domain DNA binding protein (Fig. 4, cluster C) displayed an early temporal upregulation of mRNA levels. The N-terminal region of the RAV proteins is homologous to the AP2 DNA-binding domain, while the C-terminal region exhibits homology to the highly conserved B3 domain

of VP1/ABI3 transcription factors (Kagaya *et al.*, 1999). Since EREBPs (ethylene-responsive element binding proteins) are prototype members of the AP2 family of plant transcription factors, ethylene is possibly also involved in the regulation of the RAV transcription factors. Indeed, recently Alonso et al. demonstrated that four genes that encode proteins with two plant-specific DNA binding domains, AP2 and B3, were found to be ethylene-inducible (Alonso et al., 2003b). These genes were named *ETHYLENE RESPONSE DNA BINDING FACTORS* (*EDF*). Because this analysis covered approximately 5 % of the *Arabidopsis* transcriptome and three of the isolated transcription factors displayed a clear ethylene-regulated expression pattern (the two other transcription factors were highly up-regulated in the *ein2-1* mutant only), we can speculate that as many as 60 transcription factors are involved in ethylene responses at this developmental stage.
In addition to the ethylene-regulated transcription factors, evidence was found for involvement of ethylene in post-transcriptional regulation. Previous studies suggested that the *eto1*, *eto2*, and *eto3* (ethylene overproducing) mutations, as well as treatment with cytokinin, increased ethylene biosynthesis in etiolated *Arabidopsis* seedlings via a post-transcriptional mechanism (Vogel *et al.*, 1998; Woeste *et al.*, 1999). A recent report indicated that this post-transcriptional control for all three *eto* mutants, as well as upon cytokinin treatment, is the result of an increased stability of ACS proteins (Chae *et al.*, 2003). Remarkably, in this study the transcripts of eIF5, cp29, and the RNA helicase homologue were all modulated in the same time frame (1h-2h) (Fig. 4, cluster C) of ethylene exposure. In correlation with our data, an mRNA helicase-gene, which is the orthologue of the *Arabidopsis* ATP-dependent DEAD/DEAH box helicase protein, and an ethylene-regulated elongation factor EF-Ts, for which the *Arabidopsis* functional homolog is eIF4B was found to be ethylene-regulated in tomato ripening (Zegzouti *et al.*, 1999).

2.4.3 Genes involved in cell wall metabolism

Several genes isolated in this analysis are involved in the process of cell wall metabolism. This is not surprising since the phenotypes of wild type, *ein2-1* and *ctr1-1* at this stage of development are extremely different (see Fig. 2 (A)). A putative xyloglucan endo-transglycosylase (*XET*) gene (Fig. 4, cluster F) was found to be highly upregulated in the *ein2-1* mutant. XET catalyses the cleavage and concomitant transfer of one xyloglucan molecule to another. It is thought to be involved in many aspects of cell wall biosynthesis (Arrowsmith and de Silva, 1995; Bourquin *et al.*, 2002). The putative XET protein could be involved in flexibilization of the cell wall, resulting in cell expansion. A second protein

involved in cell wall metabolism, a pectin esterase (Fig. 4, cluster B), was highly expressed in the *ctr1-1* mutant. The arabinogalactan protein (*AGP7*) gene (Fig. 4, cluster C), which encodes an extracellular hydroxyproline-rich proteoglycan, is also involved in plant cell expansion. This gene was found to be up-regulated by ethylene after 1-2h of treatment.

2.4.4 Signalling and regulatory proteins encoded by ethylene-regulated genes

Several signalling components were found to be transcriptionally regulated by ethylene (see table 3). A putative guanine nucleotide-binding protein gene (Fig. 4, cluster A) was very early up-regulated by ethylene. The involvement of GTP-binding proteins in ethylene signalling has been investigated previously (Novikova *et al.*, 1999). Recently, very rapid up-regulation of the activity of monomeric GTP-binding proteins was demonstrated in ethylene-treated rosettes of *Arabidopsis*, consistent with our data (Moshkov *et al.*, 2003). Similarly, transient up-regulation of a small GTP-binding protein, homologous to the *Arabidopsis* GTP-binding protein ara3, was demonstrated in tomato leaves after 15 minutes of exposure to ethylene (Zegzouti *et al.*, 1999). Moreover, it was suggested that G-proteins are involved in transduction chains other than that controlled by CTR1 (Hall *et al.*, 2001; Moshkov *et al.*, 2003).

2.4.5 Ethylene-regulated genes involved in defence mechanisms

Ethylene is generally known to be a regulator of responses to environmental stresses. Also in this analysis, members of stress-regulated genes were identified. Besides the known ethylene up-regulated *SAG20* gene (Fig. 4, cluster D), a Cu/Zn-superoxide dismutase copper chaperone precursor (*CCS*) (Fig. 4, cluster D) was affected by short exposure to ethylene. This protein is believed to deliver copper ions specifically to copper-zinc superoxide dismutase (CuZnSOD), thereby involved in detoxification. A microarray analysis by Cheong *et al.* (2002), demonstrated that wounding activates several genes encoding Heat Shock proteins (HSPs). Furthermore, the tomato heat-shock cognate 70 gene (*hsc 70*), orthologous to the *Arabidopsis HSP70* gene, was described to be early regulated by ethylene (Zegzouti *et al.*, 1999). In this analysis the small *HSP 17.4* (Fig. 4, cluster C) gene displayed an early ethylene-regulated expression profile. The cysteine proteinase RD21A (Fig. 4, cluster A) displayed a rather unexpected pattern in this analysis, being very early up-regulated. Previously, *RD21* was found to be up-regulated during dehydratation of *Arabidopsis* plants (Koizumi *et al.*, 1993). More recently, the *RD21* gene was reported to be senescence-associated (Weaver *et al.*, 1998). Upon ethylene

treatment a transient pattern was also observed, but the expression level peaked after 24h of treatment. Our result is not necessarily inconsistent with these data because the earliest time point included in the analysis by Weaver *et al.* (1998) was 10h of treatment with ethylene.

2.4.6 Interaction of the ethylene-signalling pathway with auxin signalling

Related to hormone signalling, a putative ACC-oxidase and a putative auxin-regulated protein were isolated as ethylene up-regulated genes. The putative ACC-oxidase gene corresponds to *ACO2*. For the *AtACO2* gene, it has been shown that the steady-state level of its transcripts increased in response to ethylene in the outer cells of the apical hook in etiolated seedlings (Raz and Ecker, 1999). In addition, up-regulation of *ACO2* is also observed in the microarray analysis of Van Zhong et al., corroborating our results.
Auxin and ethylene co-ordinately regulate several developmental programs in plants. For example, in *Arabidopsis* auxin and ethylene have been described to regulate apical hook formation, root hair elongation, root growth and hypocotyl phototropism (Harper *et al*., 2000; Lehman *et al.*, 1996; Pitts *et al*., 1998; Rahman *et al.*, 2001). In addition, auxin has been shown to stimulate ethylene biosynthesis at the level of transcription of genes encoding ACC synthase, the enzyme that forms the ethylene precursor (Abel *et al.*, 1995). Nevertheless, it is often unclear whether developmental effects attributed to auxin are solely due to this hormone or rather mediated by ethylene or resulting from a synergistic interaction between both hormones. Thus, for the ethylene-controlled putative auxin-regulated gene (Fig.4, cluster B) found in this analysis, different interpretations are possible. A positive regulation of auxin response by ethylene could occur through at least two possible pathways. The first one is by inducing production of endogenous IAA, whereas second is positive crosstalk of the signalling pathways. Since there is no prior evidence that ethylene can regulate auxin biosynthesis, the second possibility may apply.

2.4.7 Role of EIN2 and CTR1 in ethylene-independent pathways?

For cluster E and F (Fig. 4), which display a low mRNA level in the *ctr1-1* mutant and a high level in the *ein2-1* mutant, respectively, ethylene regulation is not unequivocal. For example, cluster E includes genes that are specifically down-regulated in the *ctr1-1* mutant while no effect of ethylene is seen in the wild type samples. One explanation can be that the time course of 6 hours is too short to see the effect as compared with the *ctr1-1* mutant

where ethylene signalling is constantly on. On the other hand, the *ctr1-1* mutation may affect a non-ethylene-dependent signalling pathway implying that the genes found in these clusters could be regulated through CTR-1, independent of ethylene signalling, and possibly as a result of the pleiotropic effect of the mutation. Similarly, the *ran1-3* mutant, defective in a copper transporter essential for ethylene perception, affects a non-ethylene-dependent pathway involved in cell expansion since certain morphological changes in *ran1-3* are not observed in plants grown continuously in the presence of ethylene (Woeste and Kieber, 2000). In addition, a single MAPK pathway can have multiple targets, implying that CTR1 (which encodes a MAPKKK) possibly can act as a regulator in two or more independent signal transduction pathways (Innes, 2001). Cluster F gathers genes with a high expression in all *ein2-1* samples. Again, a similar hypothesis can be proposed. Either the time period of 6 hours is too short to observe the ethylene effect on that specific gene, or the gene is affected in an ethylene-independent way in *ein2-1*. *ein2* mutants have been isolated in screens for defects in auxin transport inhibitor resistance, cytokinin response, ABA hypersensitivity, and delayed senescence (Wang *et al.*, 2002) In addition, *ein2* mutants show altered sensitivity to several bacterial and fungal pathogens. Hence, EIN2 has been proposed to lie at the crossroad of multiple hormone and stress response pathways.

2.5 CONCLUSION

This study not only identified a number of novel early genes that are regulated by ethylene, but also provided new insights into the interaction between the ethylene signal transduction pathway and other pathways. In addition, differences in responses to low and high concentrations of ethylene were observed at the level of transcript accumulation Functional analysis of the isolated genes will provide more information on the involvement of the corresponding proteins in ethylene signalling.

2.6 ACKNOWLEDGEMENTS

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2.7 EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. (ecotype Columbia-0) was purchased from Lehle seeds (Round Rock, TX). Ethylene response mutants *ctr1-1* and *ein2-1*, both in Col-0 background, were obtained at the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University. Seeds were sown under sterile conditions as described previously (Smalle *et al.*, 1997). The growth medium used was MS/2 (half-strength Murashige and Skoog; Sigma supplemented with 1% sucrose). After sowing, plates were stored at 4°C in the dark for 1 day and then put in a growth chamber at 22 °C and 60% relative humidity under white fluorescent light (Photosynthetic photon flux density (PPFD): 75μmoles/m^2 s) and long day conditions (16 h light/ 8 h dark). Plants were 19 days old at the time of ethylene exposure.

Ethylene treatments

Plants were placed inside growth chambers dedicated to gas exposures. Control plants and the plants to be treated with ethylene were placed in two adjacent identical growth cabinets. Ambient conditions were 22 °C, 60% humidity and white fluorescence light (75μmoles/m^2 s) under long day conditions (16h light/ 8h dark). 100ppb or 10ppm of ethylene in air (organic carbon free, Air Liquide Belge N.V., Aalter, Belgium) was flushed through at a flux rate of 4 refreshments per hour. In order to allow identification of very early response genes, harvesting was performed after 10 min., 20min., 30min., 1h, 2h, and 6h of treatment. Control samples treated with air were harvested after 10 min. and 6h.

RNA preparation and cDNA-AFLP analysis

Total RNA was extracted from whole plants (19 days old) using Trizol^R reagent (GIBCO/BRL, Gaithersburg, MD) according to the manufacturer's instructions. cDNA synthesis and cDNA-AFLP analysis were performed as described by (Breyne et al., 2002).

For detailed information, visit www.psb.rug.ac.be/papers/pebre/pnas.htm. The first step was the conversion of mRNA into ds cDNA using a biotinylated oligo-dT primer starting from about 10 μg total RNA. The cDNAs were digested with two restriction enzymes in a two-step reaction. After digestion with the first enzyme (*BstYI*, New England Biolabs, Beverly, MA), the 3' regions were captured on Dynabeads carrying streptavidin. Digestion with the second enzyme (*MseI*, New England Biolabs, Beverly, MA), releases the restriction fragments or transcript tags. The preamplifications were performed with the *MseI* and *BstYI* primers -with either T or C as 3' nucleotide- without selective nucleotides. From a 600-fold dilution of the pre-amplified samples, 5 μl was used for the final selective amplifications using a *BstYI* primer with 2 selective nucleotides and an *MseI* primer with 1 or 2 selective nucleotides. Amplification products were separated on 5% polyacrylamide gels using the Sequigel system (Bio-Rad, Hercules, CA). A total of 3 selective nucleotides resulted in profiles that were not too dense (the average number of tags was about 60), while the sensitivity was already high enough to detect low abundant messengers. Gels were dried on 3MM Whatman paper, exposed to Kodak Biomax Films, and scanned in a PhosphorImager 445 SI (Amersam Biosciences, Little Chalfont, UK).

Quantitative measurement of the expression profiles and data analysis

Gel images were quantitatively analysed with the AFLP-QuantarPro image analysis software (Keygene N.V., Wageningen, The Netherlands) by which all visible AFLP fragments were scored and individual band intensities were measured in each lane. The raw data were corrected for differences by using a total lane intensity correction. To that end, the intensity values were summed per lane for each primer combination and each of the sums was divided by the maximal value to yield the correction factors. Finally, all raw data were divided by these correction factors. For the corrected data, the CV value (coefficient of variation) (CV=stdev/mean) for each tag was calculated and a threshold of 0.5 was chosen for significant differentially expressed genes. The higher the CV value, the higher the differences in expression levels. Hierarchical clustering was performed using the Cluster and Treeview program (Eisen et al., 1998). Quality-based clustering was performed with a recently developed software program (De Smet *et al.,* 2002). This program is similar to K-means clustering, except that the number of clusters need not be defined in advance and the expression profiles that do not fit in any cluster are rejected. The minimal number of tags in a cluster and the required probability of genes belonging to a cluster were set to 2 and 0.95 respectively. With these parameters, all the tags were

grouped in 6 distinct clusters.

Characterization of AFLP fragments

Bands corresponding to differentially expressed genes were cut out from the gel and eluted DNA was re-amplified under the same conditions as for the selective amplification (selectivity +2/+2 or +2/+1). Sequence information was obtained either by direct sequencing of the re-amplified product with the *BstYI* or *MseI* primer or after cloning the fragments in pGEM-T easy (Promega, Madison, CA)) and sequencing of three individual clones. Only when the three sequences were identical, they were included for further analysis. The obtained sequences were compared to nucleotide and protein sequences in the publicly available databases by BLAST sequence alignments (NBLAST and TBLASTX).

Amplification of mRNA transcripts of genes in the ubiquitin-mediated degradation pathway

A 200-fold dilution of the preamplifications of the samples treated with 100ppb and 10ppm was used for each RT-PCR reaction. A series of oligonucleotide primers were designed for specific amplification of genes involved in the ubiquitin-mediated degradation pathway. The selection was primarily based on the presence of an ethylene-responsive element in the 500bp-region upstream of the start of the respective gene (list of genes presented in Ning Yan et al. (2000) and Bachmair et al. 2001). This was the case for 38 out of the 86 genes analysed. Secondly, only those transcripts containing a *BstYI* and a *MseI*-cutting site could be analysed, resulting in the analysis of 22 transcripts (See table 1). *CAD* transcript accumulation was used as an internal control as done by Moshkov et al. (2003) for the detection of early ethylene regulation (forward primer: CATGGGAGTTATCAACAATCCA, reverse primer: CATAATCCATCTTCACAACTTCG). 5μl of the diluted preamplification product was used as template in each PCR. The PCR amplification cycle that was used was 95 °C for 30s, 56 °C for 30s, 72 °C for 30s. Samples were taken after 15, 20, 25 and 27 cycles, and 20μl of the PCR product was visualized on an agarose gel. This indicated that in each case, amplifications of the target sequences were linear. For the internal control *CAD*, 30 cycles were run, according to Moshkov et al. (2003).

2.8 REFERENCES

Abel, S., Nguyen, M.D., Chow, W., and Theologis, A. (1995). ACS4, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in Arabidopsis thaliana. *J. Biol. Chem.* **270,** 19093-19099.

Abeles F.B., Morgan P.W., Saltveit M.E. Jr. (1992) *Ethylene in Plant Biology*, Ed. 2. Academis press, New York

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S., and Ecker, J.R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. *Science* **284,** 2148-2152.

Alonso, J.M., Stepanova, A.N., Solano, R., Wisman, E., Ferrari, S., Ausubel, F.M., and Ecker, J.R. (2003). Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in Arabidopsis. *Proc, Natl, Acad, Sci, U S A*, **100,** 2992-2997.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., and Ecker, J.R. (2003b). Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science **301,** 653-657.

Arrowsmith, D.A., and de Silva, J. (1995). Characterisation of two tomato fruit-expressed cDNAs encoding xyloglucan endo-transglycosylase. *Plant Mol. Biol.* **28,** 391-403.

Bachmair, A., Novatchkova, M., Potuschak, T., and Eisenhaber, F. (2001). Ubiquitylation in plants: a post-genomic look at a post-translational modification. *Trends Plant Sci.* **6,** 463-470.

Bleecker, A.B., Estelle, M.A., Sommerville, C., and Kende, H. (1988). Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science*, **241**, 1086-1089

Bourquin, V., Nishikubo, N., Abe, H., Brumer, H., Denman, S., Eklund, M., Christiernin, M., Teeri, T.T., Sundberg, B., and Mellerowicz, E.J. (2002). Xyloglucan Endotransglycosylases Have a Function during the Formation of Secondary Cell Walls of Vascular Tissues. *Plant Cell*, **14,** 3073-3088.

Breyne, P., and Zabeau, M. (2001). Genome-wide expression analysis of plant cell cycle modulated genes. *Curr. Opin. Plant Biol.* **4,** 136-142.

Breyne, P., Dreesen, R., Vandepoele, K., De Veylder, L., Van Breusegem, F., Callewaert, L., Rombauts, S., Raes, J., Cannoot, B., Engler, G., Inze, D., and Zabeau, M. (2002). Transcriptome analysis during cell division in plants. *Proc. Natl. Acad. Sci. U S* A, **99,** 14825-14830.

Callis, J., and Vierstra, R.D. (2000). Protein degradation in signalling. *Curr. Opin. Plant Biol*. **3,** 381-386.

Cancel, J.D., and Larsen, P.B. (2002). Loss-of-function mutations in the ethylene receptor ETR1 cause enhanced sensitivity and exaggerated response to ethylene in Arabidopsis. *Plant Physiol.* **129,** 1557-1567.

Chae, H.S., Faure, F., and Kieber, J.J. (2003). The eto1, eto2, and eto3 Mutations and Cytokinin Treatment Increase Ethylene Biosynthesis in Arabidopsis by Increasing the Stability of ACS Protein. *Plant Cell,* **15,** 545-559.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R. (1997). Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell*, **89,** 1133-1144.

Cheong, Y.H., Chang, H.S., Gupta, R., Wang, X., Zhu, T., and Luan, S. (2002). Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. *Plant Physiol.* **129,** 661-677.

Dahnous, K., Vigue, G.T., Law, A.G., Konsak, C.F., and Miller D.G. (1982) Height and yield response of selected wheat, barley, and triticale cultivars to ethephon. *Agro. J.* **74,** 580-582

De Smet F., Mathys J., Marchal K., Thijs G., De Moor B., and Moreau Y. (2002) . Adaptive Quality-based clustering of gene expression profiles**.** *Bioinformatics*, **18(6),** 735-746

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. U S A*, **95,** 14863-14868.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H., and Ohme-Takagi, M. (2000). Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell*, **12,** 393-404.

Goeschl, J.D., and Kays, S. (1975) Concentration dependencies of some effects of ethylene on etiolated pea, peanut, bean, and cotton seedlings*. Plant Physiol.* **55,** 670-677

Guo, H., and Ecker, J.R. (2003). Plant responses to ethylene gas are mediated by SCF(EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. *Cell* **115**, 667-677**.**

Hall M.A., Moshkov I.E., Novikova G.V., Mur L.A.J., and Smith A.R. (2001). Ethylene signal perception and transduction. *Biol. Rev.* **76**, 103-128

Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.S., Han, B., Zhu, T., Wang, X., Kreps, J.A., and Kay, S.A. (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. *Science*, **290,** 2110-2113.

Harper, R.M., Stowe-Evans, E.L., Luesse, D.R., Muto, H., Tatematsu, K., Watahiki, M.K., Yamamoto, K., and Liscum, E. (2000). The NPH4 locus encodes the auxin response factor ARF7, a conditional regulator of differential growth in aerial Arabidopsis tissue. *Plant Cell*, **12,** 757-770.

Hua, J., and Meyerowitz, E.M. (1998). Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. *Cell*, **94,** 261-271.

Hua, J., Chang, C., Sun, Q., and Meyerowitz, E.M. (1995). Ethylene insensitivity conferred by Arabidopsis ERS gene. *Science*, **269,** 1712-1714.

Hua, J., Sakai, H., Nourizadeh, S., Chen, Q.G., Bleecker, A.B., Ecker, J.R., and Meyerowitz, E.M. (1998). EIN4 and ERS2 are members of the putative ethylene receptor gene family in Arabidopsis. *Plant Cell*, **10,** 1321-1332.

Innes, R.W. (2001). Mapping out the roles of MAP kinases in plant defense. *Trends Plant Sci.* **6,** 392-394.

Johnson, P.R., and Ecker, J.R. (1998). The ethylene gas signal transduction pathway: a molecular perspective. *Annu. Rev. Genet*. **32,** 227-254.

Kagaya, Y., Ohmiya, K., and Hattori, T. (1999). RAV1, a novel DNA-binding protein, binds to bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plants. *Nucleic Acids Res*. **27,** 470-478.

Kende, H. (1993). Ethylene biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 283–307.

Kepinski, S., and Leyser, O. (2002). Ubiquitination and auxin signalling: a degrading story. *Plant Cell*, **14 Suppl,** S81-95.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R. (1993). CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases. *Cell*, **72,** 427-441.

Kim, H.S., and Delaney, T.P. (2002). Arabidopsis SON1 is an F-box protein that regulates a novel induced defense response independent of both salicylic acid and systemic acquired resistance. *Plant Cell*, **14,** 1469-1482.

Koizumi, M., Yamaguchi-Shinozaki, K., Tsuji, H., and Shinozaki, K. (1993). Structure and expression of two genes that encode distinct drought-inducible cysteine proteinases in Arabidopsis thaliana. *Gene*, **129,** 175-182.

Koyama, T., Kitajima, S., and Sato, F. (2001). Expression of PR-5d and ERF genes in cultured tobacco cells and their NaCl stress-response. *Biosci. Biotechnol. Biochem*. **65,** 1270-1273.

Larsen, P.B., and Chang, C. (2001). The Arabidopsis eer1 Mutant Has Enhanced Ethylene Responses in the Hypocotyl and Stem. *Plant Physiol*. **125,** 1061-1073.

Lehman, A., Black, R., and Ecker, J.R. (1996). HOOKLESS1, an ethylene response gene, is required for differential cell elongation in the Arabidopsis hypocotyl. *Cell,* **85,** 183-194.

Lincoln, J.E., and Fischer, R.L. (1988). Diverse mechanisms for the regulation of ethylene-inducible gene expression. *Mol. Gen. Genet.* **212,** 71-75.

Liu, Y., Schiff, M., Serino, G., Deng, X.W., and Dinesh-Kumar, S.P. (2002). Role of SCF ubiquitin-ligase and the COP9 signalosome in the N gene-mediated resistance response to Tobacco mosaic virus. *Plant Cell*, **14,** 1483-1496.

McGinnis, K.M., Thomas, S.G., Soule, J.D., Strader, L.C., Zale , J.M., Sun T.P., Steber C.M. (2003) The Arabidopsis SLEEPY1 Gene Encodes a Putative F-Box Subunit of an SCF E3 Ubiquitin Ligase. *Plant Cell* **15:** 1120-1130

Millar, A.J., and Kay, S.A. (1996). Integration of circadian and phototransduction pathways in the network controlling CAB gene transcription in Arabidopsis. *Proc. Natl. Acad. Sci. U S A*, **93,** 15491-15496.

Moshkov, I.E., Mur, L.A., Novikova, G.V., Smith, A.R., and Hall, M.A. (2003). Ethylene Regulates Monomeric GTP-Binding Protein Gene Expression and Activity in Arabidopsis. *Plant Physiol.* **131,** 1705-1717.

Novikova, G.V., Moshkov, I.E., Smith, A.R., Kulaeva, O.N., and Hall, M.A. (1999). The effect of ethylene and cytokinin on guanosine 5'-triphosphate binding and protein phosphorylation in leaves of Arabidopsis thaliana. *Planta*, **208,** 239-246.

Ouaked, F.,,Rozhon, W., Lecourieux, D., and Hirt, H. (2003) A MAPK pathway mediates ethylene signalling in plants. *Embo. J.* **22:** 1282-1288

Park, J.H., Oh, S.A., Kim, Y.H., Woo, H.R., and Nam, H.G. (1998). Differential expression of senescence-associated mRNAs during leaf senescence induced by different senescence-inducing factors in Arabidopsis. *Plant Mol. Biol.* **37,** 445-454.

Pitts, R.J., Cernac, A., and Estelle, M. (1998). Auxin and ethylene promote root hair elongation in Arabidopsis. *Plant J.* **16,** 553-560.

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C., and Genschik, P. (2003). EIN3-dependent regulation of plant ethylene hormone signaling by two arabidopsis F box proteins: EBF1 and EBF2. *Cell* **115**, 679-689.

Rahman, A., Amakawa, T., Goto, N., and Tsurumi, S. (2001). Auxin is a positive regulator for ethylene-mediated response in the growth of Arabidopsis roots. *Plant Cell Physiol.* **42,** 301-307.

Raz, V., and Ecker, J.R. (1999). Regulation of differential growth in the apical hook of Arabidopsis. *Development*, **126,** 3661-3668.

Roman, G., Lubarsky, B., Kieber, J.J., Rothenberg, M., and Ecker, J.R. (1995). Genetic analysis of ethylene signal transduction in Arabidopsis thaliana: five novel mutant loci integrated into a stress response pathway. *Genetics*, **139,** 1393-1409.

Sakai, H., Hua, J., Chen, Q.G., Chang, C., Medrano, L.J., Bleecker, A.B., and Meyerowitz, E.M. (1998). ETR2 is an ETR1-like gene involved in ethylene signalling in Arabidopsis. *Proc. Natl. Acad. Sci. U S A*, **95,** 5812-5817.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., and Manners, J.M. (2000). Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. *Proc. Natl. Acad. Sci. U S A*, **97,** 11655-11660.

Smalle, J., Haegman, M., Kurepa, J., Van Montagu, M., and Van Der Straeten, D. (1997). Ethylene can stimulate Arabidopsis hypocotyl elongation in the light. *Proc. Natl. Acad. Sci. U S A*, **94,** 2756-2761.

Smalle, J., Kurepa, J., Yang, P., Babiychuk, E., Kushnir, S., Durski, A., and Vierstra, R.D. (2002) Cytokinin growth responses in Arabidopsis involve the 26S proteasome subunit RPN12. *Plant Cell* **14:** 17-32

Smalle, J., Kurepa, J., Yang, P., Emborg, T.J., Babiychuk, E., Kushnir, S., and Vierstra, R.D. (2003) The pleiotropic role of the 26S proteasome subunit RPN10 in Arabidopsis growth and development supports a substrate-specific function in abscisic acid signalling. *Plant Cell* **15:** 965-980

Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R. (1998). Nuclear events in ethylene signalling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes Dev*. **12,** 3703-3714.

Suzuki, G., Yanagawa, Y., Kwok, S.F., Matsui, M., and Deng, X.W. (2002). Arabidopsis COP10 is a ubiquitin-conjugating enzyme variant that acts together with COP1 and the COP9 signalosome in repressing photomorphogenesis. *Genes Dev.* **16,** 554-559.

Trentmann, S.M. (2000). ERN1, a novel ethylene-regulated nuclear protein of Arabidopsis. *Plant Mol. Biol.* **44,** 11-25.

Van Zhong, G., and Burns, J.K. (2003). Profiling ethylene-regulated gene expression in Arabidopsis thaliana by microarray analysis. *Plant Mol. Biol.* **53,** 117-131.

Vogel, J.P., Woeste, K.E., Theologis, A., and Kieber, J.J. (1998). Recessive and dominant mutations in the ethylene biosynthetic gene ACS5 of Arabidopsis confer cytokinin insensitivity and ethylene overproduction, respectively. *Proc. Natl. Acad. Sci. U S A*, **95,** 4766-4771.

Wang, H., Brandt, AS,, and Woodson, W.R. (1993) A flower senescence-related mRNA from carnation encodes a novel protein related to enzymes involved in phosphonate biosynthesis. *Plant Mol. Biol.* **22:** 719-724

Wang, K.L., Li, H., and Ecker, J.R. (2002). Ethylene biosynthesis and signalling networks. *Plant Cell*, **14 Suppl,** S131-151.

Weaver, L.M., Gan, S., Quirino, B., and Amasino, R.M. (1998). A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. *Plant Mol. Biol*. **37,** 455-469.

Woeste, K.E., and Kieber, J.J. (2000). A strong loss-of-function mutation in RAN1 results in constitutive activation of the ethylene response pathway as well as a rosette-lethal phenotype. *Plant Cell*, **12,** 443-455.

Woeste, K.E., Ye, C., and Kieber, J.J. (1999). Two Arabidopsis mutants that overproduce ethylene are affected in the posttranscriptional regulation of 1-aminocyclopropane-1-carboxylic acid synthase. *Plant Physiol*. **119,** 521-530.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D., and Xie, D. (2002). The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. *Plant Cell*, **14,** 1919-1935.

Yan, N., Doelling, J.H., Falbel, T.G., Durski, A.M., and Vierstra, R.D. (2000). The ubiquitin-specific protease family from Arabidopsis. AtUBP1 and 2 are required for the resistance to the amino acid analog canavanine. *Plant Physiol.* **124,** 1828-1843.

Zegzouti, H., Jones, B., Frasse, P., Marty, C., Maitre, B., Latch, A., Pech, J.C., and Bouzayen, M. (1999). Ethylene-regulated gene expression in tomato fruit: characterization of novel ethylene-responsive and ripening-related genes isolated by differential display. *Plant J*. **18,** 589-600.

Zhou, J., Tang, X., and Martin, G.B. (1997). The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a cis-element of pathogenesis-related genes. *Embo J*. **16,** 3207-3218.

Chapter 3

Physiological analysis of SALK lines carrying an insertion in ethylene-modulated genes

Chapter 3: Physiological analysis of SALK lines carrying an insertion in ethylene-modulated genes

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3.1 SALK-MUTANTS

The cDNA-AFLP approach yielded a collection of genes with potential function shown to be early regulated by ethylene. In addition, for a number of genes, no function is known yet. A tool for the functional analysis of the role of these genes in ethylene signalling is the analysis of loss-of-function mutations in these genes.

The Salk Institute Genome Analysis Laboratory (SIGNAL) used high-throughput genome sequencing methods to identify the sites of insertion of Agrobacterium T-DNA in the *Arabidopsis* genome to create a collection of sequence-indexed insertion mutants. The data are made available via a web accessible graphical interface-T-DNAExpress-(http://signal.salk.edu/cgi-bin/tdnaexpress) that provides both text and a searchable database of the DNA sequences flanking the insertions. All DNA sequences are deposited into GenBank (http://www.ncbi.nlm.nih.gov/) and are also provided to The *Arabidopsis* Information Resource TAIR (http://www.arabidopsis.org/). Seeds from the T-DNA insertion lines are deposited at the *Arabidopsis* Biological Resource Center (ABRC) (http://www.arabidopsis.org/abrc/at) Ohio State University. A recent report mentioned that the set of 88.122 T-DNA insertions covers about 74% of all *Arabidopsis* genes (Alonso et al., 2003).

By searching the sequence-indexed insertion mutant database, we were able to identify insertion mutants for 40 genes from our collection (see table 1). The T-DNA insertion was confirmed by growing the plants on kanamycin (50mg/ml) and by amplifying the *NptII*-gene which encodes the kanamycin resistance. For some of the mutants we also amplified the flanking region of the insert to investigate the zygosity of the mutation. For this purpose, we made use of two gene-specific primers, covering the place of the insert, and the left-border primer of the T-DNA insert. By comparing the PCR fragments generated by the left-border primer with one gene specific primer an that obtained by the combination of gene specific primers, homo-and heterozygotes could be discerned.

To test the ethylene response for these mutants, we evaluated their hypocotyl growth on ACC in the light and the dark. In addition, the effect of ACC on root length of 2 weeks-old plants was measured. Finally, for some mutants the root growth in the dark on 5-days old

Table 1: Overview of tested SALK-lines. All seeds are ordered from ABRC. For each tDNA-inserted SALK-line, the function, locus number, and the amount of independent Km-resistant lines are listed. The results of the PCR-amplification of the *NptII*-gene and the zygosity-test for each individual line is indicated (empty box = not tested yet).

stock number	locus number	Km-resistant	gene or protein	confirmation Npt2	zygosity
SALK_010894	At1g02840	1	splicing factor SF2	present	homozygous
		2		present	homozygous
		3		present	homozygous
		4		present	homozygous
		5		present	homozygous
		6		present	hemizygous
		7		present	homozygous
SALK_040709	At4g04020	1	putative fibrillin	present	
		2		present	
		3		present	
		4		present	
SALK_010311	At2g45210	1	putative auxin-regulated gene	not present	
		2		present	homozygous
SALK_015908	At2g27290	1	unknown protein	present	homozygous
		2		present	homozygous
		3		present	homozygous
		4		present	homozygous
		5		not present	
SALK_031088	At4g39090	1	cysteine proteinase RD19A	present	homozygous
		2		present	hemizygous
		3		present	homozygous
		4		present	
		5		present	hemizygous
		6		present	homozygous
SALK_053577	At4g39090	1	cysteine proteinase RD19A	present	hemizygous
		2		present	hemizygous
		3		present	hemizygous
SALK_057834	At2g33370	1	ribosomal protein L23	not present	
SALK_044146	At1g62380	1	putative ACC oxidase	present	homozygous
SALK_000234	At4g22950	1	MADS box protein AGL14	present	homozygous
		2		not present	homozygous
SALK_048446	At1g36730		eukaryotic translation initiation factor 5 -related		
SALK_016325	At2g18960	no Km-res.plant	plasma membrane proton ATPase (PMA)		
SALK_042796	At3g13450	1	branched chain alpha-keto acid dehydrogenase E1 beta subunit (din4)	present	homozygous
		2		present	homozygous
SALK_037263	At2g27290	no Km-res.plant	unknown protein		
SALK_040413	At5g02020	no Km-res.plant	putative protein		
SALK_065256	At1g47128	1	cysteine proteinase RD21A	present	homozygous
		2		present	homozygous
		3		present	homozygous
		4		present	homozygous
SALK_068605	At5g22500	1	male sterility protein	present	homozygous
		2		present	homozygous
		3		present	homozygous
		4		present	homozygous
		5		present	homozygous
		6		present	homozygous
		7		present	homozygous
		9		present	homozygous
		8		present	homozygous
SALK_065559	At3g53460	1	RNA-binding protein cp29	present	homozygous
		2		present	homozygous
		3		present	homozygous
		4		present	homozygous
		5		present	homozygous
		6		present	homozygous
		7		present	homozygous
		8		present	homozygous
SALK_055112	At2g35340	1	RNA helicase	present	homozygous
		2		present	homozygous
		3		present	homozygous
		4		not present	no band
SALK_002025	At5g39190	1	germin-like protein	present	homozygous
		2		present	homozygous
		3		present	homozygous
		4		present	homozygous
SALK_013620	At4g39090	1	cysteine proteinase RD19A	present	homozygous
		2		present	homozygous
		3		present	homozygous
SALK_005495	At1g05230	1	ovule-specific homeotic protein	present	homozygous
		2		present	homozygous
		3		not present	no band
		4		present	homozygous
		5		not present	homozygous
		6		present	homozygous
		7		present	homozygous
SALK_005489	At1g05230	1	ovule-specific homeotic protein	not present	no band
		2		present	homozygous
		3		present	homozygous
		4		present	homozygous
SALK_051162	At1g63800	1	UBC5	present	homozygous
		2		present	homozygous
		3		present	homozygous
		4		present	homozygous
SALK_069662	At2g18800	1	putative xyloglucanendotransglycosylase	present	homozygous
		2		present	homozygous
		3		present	homozygous
		4		present	homozygous
		5		present	homozygous
SALK_003562	At3g49220	1	pectineesterase -like protein	present	homozygous
		2		present	homozygous
		1'		not present	no band
		2'		present	homozygous

stock number	locus number	Km-resistant	gene or protein	confirmation Npt2	zygosity
SALK_009522	At1g75960	1	AMP-binding protein	present	homozygous
SALK_055487	At5g01600	1	ferritin precursor	present	hemizygous
		2		present	hemizygous
		3		present	hemizygous
		4		present	no band
		5		present	homozygous
		6		present	homozygous
		7		present	hemizygous
		8		present	homozygous
SALK_065256	At1g47128	1	cysteine proteinase RD21A	present	homozygous
		2		present	homozygous
		3		present	homozygous
		4			
SALK_049352	At1g01560	1	MPK5		
SALK_032664	At3g13750	no Km-res.plant	bGAL1		
SALK_064139	At5g25040	1	putative membane protein	present	
		2		present	
		3		present	
SALK_074725	At1g06510	1	hypothetical protein	present	
		2		not present	
		3		present	
		4		present	
		5		present	
		6		present	
		7		present	
		8		present	
		9		present	
		10		present	
		11		present	
		12		present	
		13		present	
		14		present	
		15		present	
		16		present	
		17		present	
		18		present	
SALK_071319	At1g68600	1	expressed protein	present	
		2		present	
		3		present	
		4		present	
		5		present	
SALK_055116	At4g26130	1	unknown protein	not present	
		2		present	
		3		present	
		4		present	
		5		present	
SALK_016739	At1g67830	1	iEP4	present	
		2		present	
		3		present	
		4		present	
		5		present	
		6		present	
		7		present	
		8		present	
		9		present	
		10		present	
		11		present	
SALK_008497	At1g80440	1	unknown protein	present	
		2		present	
		3		present	
		4		present	
		5		present	
		6		present	
		7		present	
		8		present	
		9		present	
		10		present	
		11		present	
		12		present	
		13		present	
SALK_061320	At5g58100	1	putative protein	present	
		2		present	
SALK_035202	At3g58490	no Km-res.plant	putative protein		
SALK_088298	At5g66880	1	protein kinase	present	
		2		present	
		3		present	
		4		present	
		5		present	
		6		present	
		7		present	
		8		present	
SALK_003938	At3g10130	1	unknown protein	present	
		2		present	
		3		present	
		4		present	
		5			
		6			
SALK_036324	At5g65390	1	AGP7	present	
		2		present	
		3		present	
		4		present	
		5		present	
		6		present	

stock number	locus number	Km-resistant	gene or protein	confirmation Npt2	zygosity
SALK_049458	At3g10130	1	unknown protein	present	
		2		present	
		3		present	
		4		present	
		5		present	
		6		present	
SALK_074351	At3g14810	no Km-res.plant	hypothetical protein		
SALK_049808	At4g29930	1	unknown protein	present	
		2		present	
		3		present	
		4		present	
		5		present	
SALK_034132	At1g53480	1	unknown protein	present	
		2		present	
		3		present	
		4		present	
		5		present	
		6		present	
SALK_071767	AT5g05250	1	expressed protein	present	
		2		present	
		3		present	
		4		present	
		5		present	
		6		present	
		7		present	
		8		present	
		9		present	
SALK_017898	At5g54020	1	CHP-rich zinc finger protein, putative	present	
		2		present	
		3		present	
SALK_065288	At2g18960	1	plasma membrane proton ATPase (PMA)	present	
		2		present	
		3		present	
		4		present	
		5		present	
		6		present	
SALK_031519	At3g14940	1	phosphoenolpyruvate carboxylase (PPC)	present	
		2		present	
		3		present	
		4		present	
		5		present	
SALK_045132	At5g54020	1	putative CHP-rich zinc finger protein	present	
		2		present	
		3		present	
		4			
		5		present	
SALK_003066	At3g53460	1	RNA-binding protein cp29	present	
SALK_003937	At5g39190	1	germin-like protein (GLP2a)	not present	
		2		present	
SALK_003938	At3g10130	1	unknown protein	present	
		2		present	
		3		present	

seedlings was investigated. For the majority of the T-DNA insertion mutants no detectable alterations in morphology were observed. Several reasons can be invoked for these observations. First of all, there is the problem of redundancy. Many genes are part of a gene family within which functional overlap is possible. In addition, the isolated ethylene-regulated genes could be functioning in only one branch of the ethylene responses and not in the responses in seedlings and 2 week old plants which were investigated. Since the genes are isolated from ethylene-treated plants at 19 days of age, their response could be specific to a role of ethylene in vegetative development, senescence, or different ethylene-induced stress responses which have not been investigated. Furthermore, the genes were recovered as early ethylene-regulated genes. Therefore, their role in ethylene signalling could be specific for a short-term response, for example activation or repression of a certain pathway or the activity of other proteins which do not lead to significant alterations in morphology. Despite the fact that most T-DNA insertion mutants displayed a normal phenotype, some of them showed altered responses to ACC. The observed changes were

usually small but significant (see results of the student T- test).

3.2 HYPOCOTYL RESPONSE IN THE LIGHT ON LNM

For the hypocotyl elongation on ACC in the light, three insertion mutants displayed a less pronounced response than the wild type. In figure 1 A-C each mutant is presented with their corresponding wild type control. The mutants in panel A-B have their insert in genes which encode presently unknown proteins. The function of the gene in insertion line 0549808 in panel C corresponds to a putative bHLH protein. The expression patterns of the two unknown proteins indicated a higher expression in the *ctr1-1* mutant. Therefore one of the possibilities is that the tDNA-insertion in these mutants could disrupt ethylene responsiveness, resulting in a less pronounced response to ACC. On the other hand, the expression of the putative bHLH protein was highly up-regulated in the *ein2-1* mutant. Therefore in this case the tDNA-insertion could be specific to a domain in the gene which is normally under negative control regulation by ethylene.

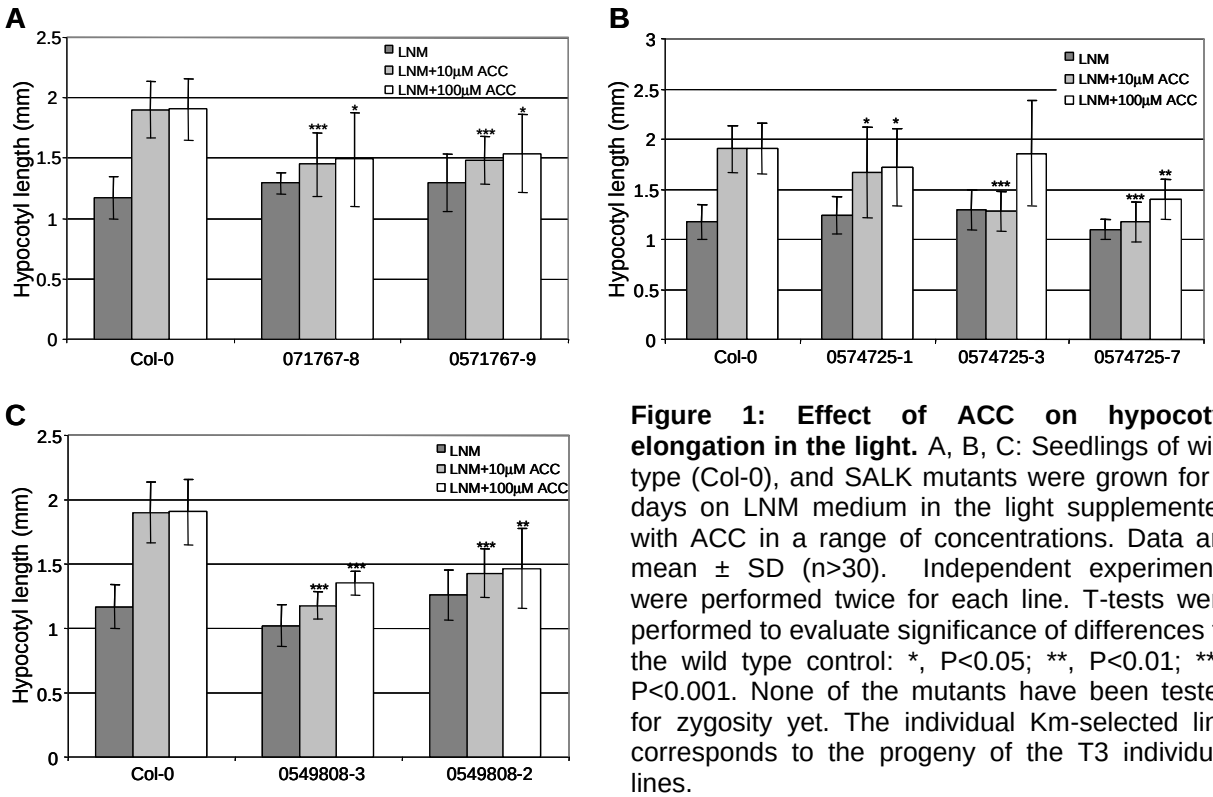


Figure 1: Effect of ACC on hypocotyl elongation in the light. A, B, C: Seedlings of wild type (Col-0), and SALK mutants were grown for 8 days on LNM medium in the light supplemented with ACC in a range of concentrations. Data are mean ± SD (n>30). Independent experiments were performed twice for each line. T-tests were performed to evaluate significance of differences to the wild type control: *, P<0.05; **, P<0.01; ***, P<0.001. None of the mutants have been tested for zygosity yet. The individual Km-selected line corresponds to the progeny of the T3 individual lines.

3.3 HYPOCOTYL RESPONSE IN THE DARK ON LNM

When investigating the response of hypocotyls in the dark on GM with and without ACC, two mutants appeared to have significant differences in their response as compared to

wild type. One insertion mutant was less sensitive to ACC (Fig. 2A), one appeared to be more sensitive (Fig.2B). The results are displayed in figure 2 A-B. The mutant in panel A has an insertion in the gene encoding the MADS box protein AGL14. This gene displayed a higher expression in the *ein2-1* mutant. The mutant in graph B has its insertion in a gene encoding an unknown protein. The expression pattern of this gene was characterized by a peak after two hours of treatment. In both mutants a crucial domain of ethylene regulation could be affected.

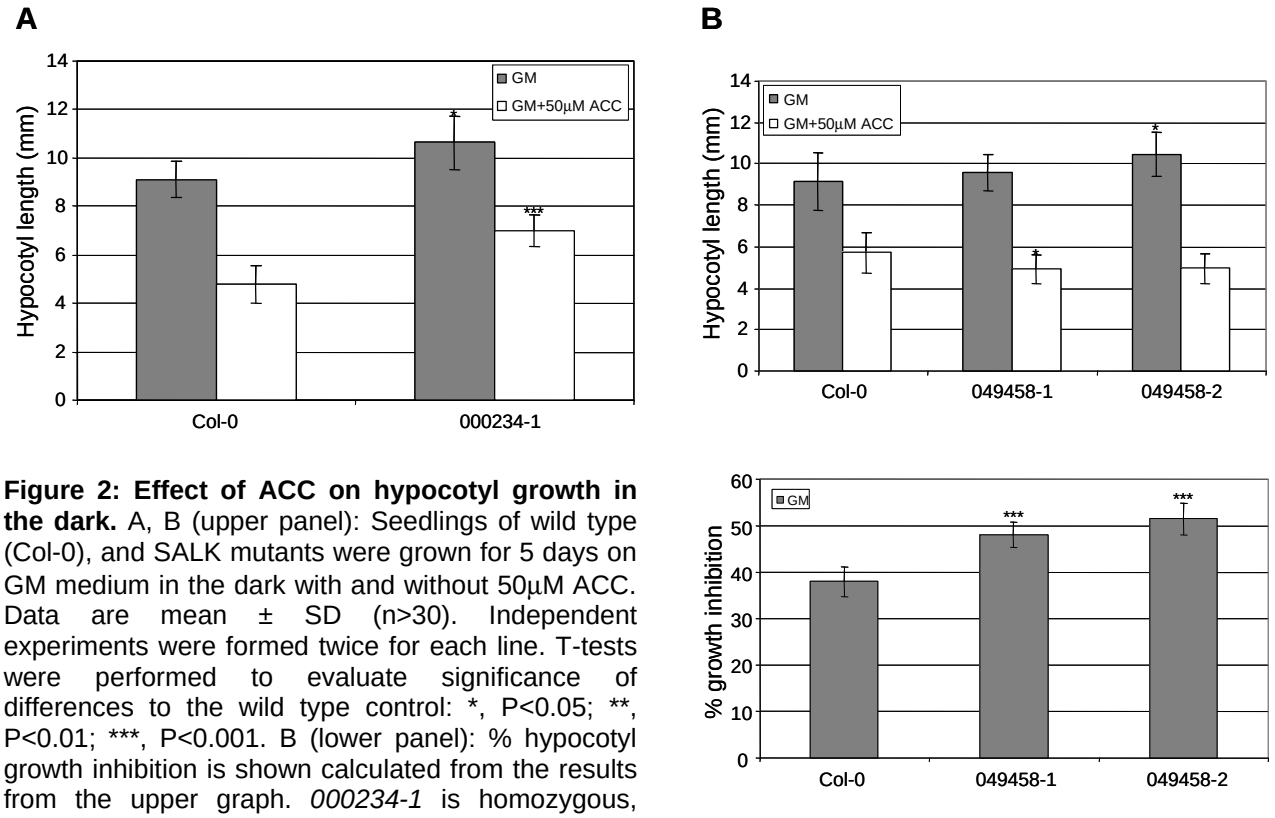


Figure 2: Effect of ACC on hypocotyl growth in the dark. A, B (upper panel): Seedlings of wild type (Col-0), and SALK mutants were grown for 5 days on GM medium in the dark with and without 50µM ACC. Data are mean ± SD (n>30). Independent experiments were formed twice for each line. T-tests were performed to evaluate significance of differences to the wild type control: *, P<0.05; **, P<0.01; ***, P<0.001. B (lower panel): % hypocotyl growth inhibition is shown calculated from the results from the upper graph. *000234-1* is homozygous, 0*49458-1/2* have not been tested for zygosity yet. The individual Km-selected line corresponds to the progeny of the T3 individual lines.

3.4 ROOT RESPONSE IN THE LIGHT ON GM

A third ethylene response test was the root inhibition of 2 weeks old plants in the light on medium with ACC. In this case, two insertion mutants were behaving significantly different from the wild type. Both were less sensitive to ACC than the wild type. In figure 3 A-B, the response of the individual insertion mutants is shown with their corresponding wild type control. The gene of interest for the mutant in panel A encodes the RNA–binding protein cp29. The pattern of expression of this gene peaked after 1h-2h of treatment. The mutant in panel B has its insertion the gene encoding the MADS-box protein AGL14. Interestingly, this insertion mutant also behaved differently in the test for hypocotyl growth in the dark.

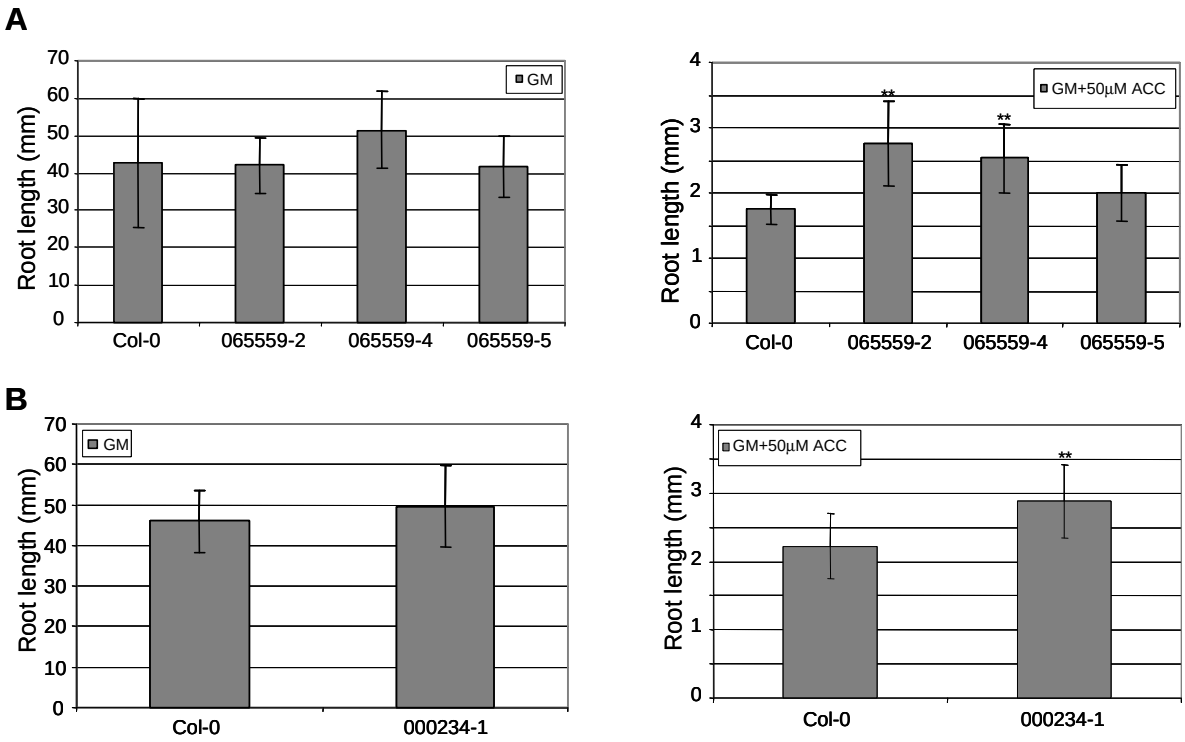


Figure 3: Effect of ACC on root growth in the light. A, B: Seedlings of wild type (Col-0), and SALK mutants were grown for two weeks on GM medium in the light with (right panel) and without 50µM ACC (left panel). Data are mean ± SD (n>30). Independent experiments are performed twice for each line. T-tests are performed to evaluate significance of differences to the wild type control: *, P<0.05; **, P<0.01; ***, P<0.001 *N500234-1* is homozygous, *N0065559-2/4/5* have not been tested for zygosity yet. The individual Km-selected line corresponds to the progeny of the T3 individual lines. .

3.5 PRELIMINARY CONCLUSIONS

Altogether, we can conclude that for 6 out of 40 available SALK mutants containing mutations in genes identified in the cDNA-AFLP profiling, a role in ethylene signalling is confirmed by functional analysis of the respective T-DNA insertion lines. Although these results are promising, further confirmation is needed. At present, only one of the 6 lines is confirmed to have the T-DNA inserted in the right gene by amplification of the flanking DNA sequences. In addition, due to the redundancy of genes within a family, knocking out multiple members of a family or crosses between single-mutants, could provide additional information on the role of these genes in ethylene function.

3.6 ACKNOWLEDGEMENTS

This work was done in collaboration with Cedric De Smet. More detailed information of all measurements can be viewed in the undergraduate thesis of Cedric De Smet (2002-2003)

with the title “Karakterisering van mutanten in de ethyleenrespons bij *Arabidopsis* thaliana”.

3.7 EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

All SALK mutants originated from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University. Columbia (Col-0) was purchased from Lehle seeds (Round Rock, TX). Conditions of the growth chamber and greenhouse were 22°C and 60% relative humidity with white fluorescent light (75 µmol/m^2 per s) and long day conditions (16 h light/8 h dark).

Media and treatments

The *Arabidopsis* seedlings were grown under sterile conditions as described in Smalle *et al*. (1997). The rich medium used was GM (Growth Medium) supplemented with 0.5 g/L of MES (Roman *et al.*, 1995). ACC (1-amino cyclopropane-1-carboxylic acid) was obtained from Sigma-Aldrich (St. Louis, MO). Kanamycin originated from Duchefa (Harlem, The Netherlands). The hormone and antibiotic solution was added to the medium after filter sterilization. Plates were stored at 4°C in the dark for 2 days and then put in a growth chamber.

Analysis of inserts

Per individual Km-selected line, DNA was prepared from a single leaf with a single-step protocol (Edwards et al., 1991). 5µl of genomic DNA was used as template in each PCR. For some of the mutants the flanking region of the insert was amplified to investigate the zygosity of the mutation. Two gene-specific primers, covering the place of the insert, and the left-border primer of the T-DNA insert were used. The PCR fragments generated by the left-border primer with one gene specific primer an that obtained by the combination of gene specific primers were compared to discern homo-and heterozygotes. PCR was performed using the following conditions: 94 °C for 30s, 55 °C for 30s, 72 °C for 30s (35 cycles). 20µl of the PCR product was visualized on a 1.2% agarose gel. The following primers were used: *NptII:* forward primer: 5’-AGACAATCGGCTGCTCTGAT-3’, reverse primer:

5’-CAATAGCAGCCAGTCCCTTC-3’; LB-primer: 5’GCGTGGACCGCTTGCTGCAACT-3’; SALK_00234: forward primer: 5’-CTCAAGCTTCGGTCCAACTG-3’, reverse primer: 5’- GCGAGAGACGAAACATCTGG- 3’.

Biometric analysis

Measurements of hypocotyl length of light grown seedlings were done as in Smalle et al. (1997) using a Stemi SV 11 microscope equipped with a graduated ocular (Zeiss, Jena, Germany).

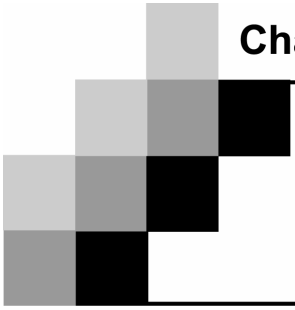
3.8 REFERENCES

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., and Ecker, J.R. (2003). Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science **301,** 653-657.

Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res **19,** 1349.

Roman, G., and Ecker, J.R. (1995). Genetic analysis of a seedling stress response to ethylene in Arabidopsis. Philos Trans R Soc Lond B Biol Sci **350**, 75-81

Smalle, J., Haegman, M., Kurepa, J., Van Montagu, M., and Van Der Straeten, D. (1997). Ethylene can stimulate Arabidopsis hypocotyl elongation in the light. Proc Natl Acad Sci U S A **94,** 2756-2761.



Chapter 4

Transcript profiling by Microarray analysis reveals novel insights into the early response to ethylene in *Arabidopsis*

Chapter 4: Transcript profiling by microarray analysis reveals novel insights into the early role of ethylene in *Arabidopsis*.

Annelies De Paepe, Marnik Vuylsteke, Paul Van Hummelen, and Dominique Van Der Straeten

4.1 SUMMARY

A comprehensive transcriptome analysis by means of a cDNA-microarray was performed in order to gain further understanding of the molecular mechanisms of immediate ethylene action. Col-0 plants were treated with exogenous ethylene for 6 different time-points ranging from 10 minutes to 6 hours. The ethylene-insensitive mutant *ein2-1* was analyzed in parallel. Out of the 6008 genes present on the chip, 214 genes showed significant differential expression during the time course. The cluster analysis and functional classification allowed us to determine classes of co-ordinately regulated genes under ethylene control. In particular, a large number of genes involved in cell rescue, disease and defence mechanisms were identified as early ethylene-regulated genes. Furthermore, the data provide insight into early regulatory steps of ethylene signalling and ethylene-regulated transcription and protein fate. The results also reveal novel understanding of the integration of the ethylene pathway with other signals. Of particular interest is the overlap between ethylene response and responses to ABA, sugar and auxin. In conclusion, the data provides deeper insight into early regulatory steps of ethylene signalling.

4.2 INTRODUCTION

Ethylene is a simple two-carbon gaseous molecule with profound effects on plant growth and development, including promotion of phenomena such as the seedling triple response, response to pathogen attack, fruit ripening, and tissue senescence (Johnson and Ecker, 1998). The current knowledge of the regulation of ethylene signalling in *Arabidopsis* has emerged from genetic studies on mutants either fully or partially defective in ethylene responses (*etr*, *ein)* or with constitutive characteristics (*ctr*), providing a view on the mechanism underlying ethylene signalling (Bleecker et al., 1988, Guzman and Ecker, 1990; Kieber et al., 1993; Roman et al., 1995). A linear pathway was proposed that initiates with ethylene binding at a family of ethylene receptors and terminates in a transcriptional cascade (Chang, 2003). In addition to the identification of receptors and downstream signalling components, these studies also have indicated the importance of

transcriptional regulation in ethylene responses.

Although genetic screens were originally designed to identify specific components in ethylene signalling, mutations in these genes often confer changes in sensitivity to other hormones as well. For instance, mutations in ethylene signalling components have been recovered not only in screens using auxin transport inhibitors or cytokinins, but also in screens for suppressor and enhancer mutations of abscisic acid (ABA) mutants and for regulators of sugar metabolism (Vogel et al., 1998; Zhou et al., 1998; Beaudoin et al., 2000; Ghassemian et al., 2000). Together, these facts indicate that the linear representation of the hormone signalling pathways controlling a specific aspect of plant growth and development is oversimplified, and that hormones interact with each other and with a plethora of developmental and metabolic signals. Therefore, dissecting crosstalk between ethylene and other pathways is critical to the understanding of a plant's response to ethylene. To this end, comparing genetic interaction maps with patterns based on transcript profiling and other genomic technologies may create a more representative view of hormone interactions within the cell. Recent research using cDNA microarrays revealed an important overlap of genes regulated by jasmonic acid, ethylene, and upon infection with an avirulent pathogen, indicating a network of regulatory interactions and coordination during pathogen and wounding responses (Schenk et al., 2000). Moreover, a transcriptional profiling of genes in response to wounding identified a number of genes involved in ethylene signalling (Cheong *et al.*, 2002).

An extensive kinetic analysis of the transcriptional cascade in response to ethylene would allow mapping of the interaction between ethylene and other pathways in a comprehensive manner at the genomic level, besides identification of novel genes involved in ethylene signalling. We previously performed a pilot study using the cDNA-amplified fragment length polymorphism (AFLP) transcript profiling approach in order to gain further understanding of the molecular mechanisms of immediate early ethylene action (described in Chapter 2, page 45). A set of ethylene-responsive genes were isolated, falling in different classes of expression pattern and including a number of novel genes which belong to diverse functional groups. In this study, we made use of cDNA arrays spotted with 6008 unique cDNAs from *Arabidopsis* (WWW.microarray.be), to extend the collection of previously identified ethylene-regulated genes. The changes in the *Arabidopsis* transcriptome were monitored during early time points after ethylene treatment. This was done both in wild type *Arabidopsis* plants and in the ethylene-insensitive mutant *ein2-1* over a time course from 10 minutes until 6 hours after ethylene treatment, allowing a clear distinction between immediate early , early, and later ethylene responses.

Altogether 214 genes were significantly modulated over time. In particular, a large number of genes involved in cell rescue, disease and defence mechanisms were identified as early ethylene-regulated genes. Furthermore, the data provide insight into early regulatory steps of ethylene signalling and ethylene-regulated transcription and protein fate. Novel players in the interaction between ethylene and several other signals have been identified by this study.

4.3 RESULTS

4.3.1 6K-*Arabidopsis* microarray: experimental set-up, validation of data and data analysis

A microarray containing 6008 unique *Arabidopsis* ESTs spotted in duplicate (referred to as 6K microarray), representing about a fourth of the *Arabidopsis* transcriptome, was used to monitor the transcriptome changes in *Arabidopsis* wild type (Col-0) and mutant plants (*ein2-1*) upon a 6h treatment with 10ppm ethylene. Individual wild type plants and mutant plants were harvested after 0, 10, 20, 30, min, 1, 2, and 6h of treatment. A kinetic analysis with multiple sequential time points increases the temporal resolution and thus allows discrimination between immediate early, early and late ethylene response genes. The analysis of the ethylene-insensitive mutant *ein2-1*, allowed us to distinguish between truly ethylene responsive genes from genes influenced by circadian rhythm or by mechanical stress during the treatment. A reference design was used, consisting of dye-swap experiments (labelling with Cy3-Cy5, see experimental procedures) wherein test samples (Col-0 and *ein2-1* sampled across the seven time points) are compared to the reference sample *ctr1-1*, accounting for a total of 28 cDNA microarrays**.** Our raw data consisted of 112 (14 x 2 (duplicate spots) x 2 (dye-swap)) measurements for each of the 6008 *Arabidopsis* genes. We excluded 749 (12.5%) clones from the analysis for which no 'consistent' (see experimental procedures) positive signal for the two test samples Col-0 and *ein2-1* across the seven time points was detected.

We applied two sequential mixed model analyses of variance (ANOVAs) (Wolfinger et al., 2001) to the base-2 logarithm of all the "lowess"-transformed spot measurements, using the REML method (see experimental procedures). In a first step, a general linear normalization model was used to estimate the global variation of the collection of 5259 cDNA fragments in the form of random array effects, random channel effects and random error. In a second step, 5259 gene-specific models were applied to partition the gene-specific variation into fixed sample, genotype, time, genotype-specific response in time

effects, random spot effects and random error. To test expression differences between genotype, time points, and the interaction term genotype x time, we used Wald statistics, which should follow approximate X^2-distribution under the null hypothesis with degrees of

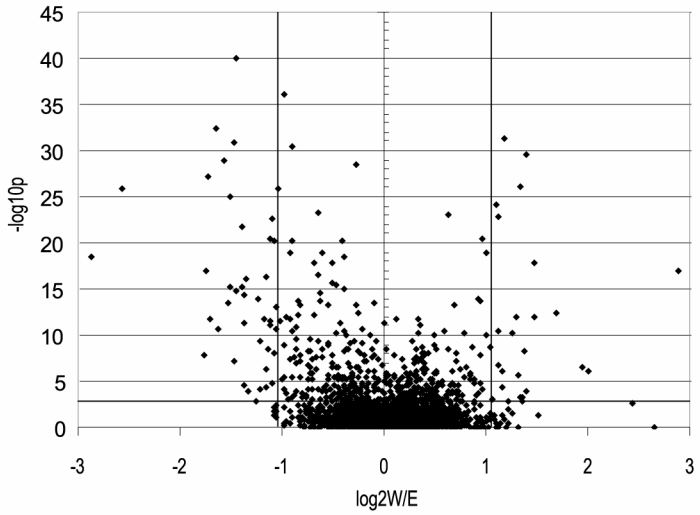


Figure 1: Volcano plot: the negative log10 of the *p* value from the Wald statistic against the log2 ratio of the means of wild type (W) to *ein2-1* (E). The horizontal line represents the threshold of *p*=0.001. The two vertical lines indicate a two-fold cut-off for either repression or induction.

freedom (df) equal to 1, 6, and 6, respectively. The cut-off selection for differentially expressed genes was based on two criteria. A first cut-off was set at $p<0.001$ (Fig. 1) and no further adjustments for multiple testing were made. At this level of significance, 476, 1368, and 231 genes were differentially expressed between genotypes (G), time points (T) and genotype x time (GT), respectively (Fig. 2). Genes only differentially expressed over time (T only) were considered as affected by circadian rhythm and were therefore ruled out for the further analysis.

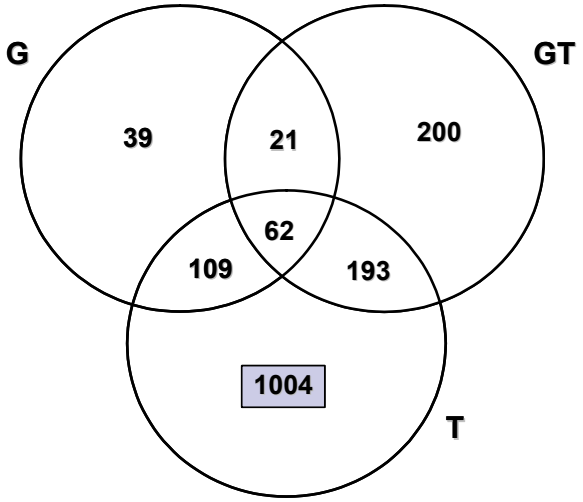


Figure 2: Venn diagrams of the numbers of overlapping and nonoverlapping genes with a significance level of $p< 0.001$ between genotypes (G), time points (T), and genotype x time (GT). Boxed number indicates the amount of genes only differentially expressed over time, these genes were considered as affected by circadian rhythm

The second criterion was a two-fold change in expression for the treated samples compared to control samples, or for *ein2*- average values compared to wild type-average values. A group of 214 significantly ethylene regulated genes remained from this dual selection (see additional data, 4.9, p 110). The strongest effect of time on gene expression was a 12-fold change between two time points, 39 genes showed changes in expression higher than 5-fold, and 106 genes higher than 3-fold. In addition, for 12 out of the selected 214 significant differentially expressed genes two independent cDNA clones were spotted on the array. The expression profiles were identical for every gene, indicating a high reproducibility of the hybridization signal (Fig. 3).

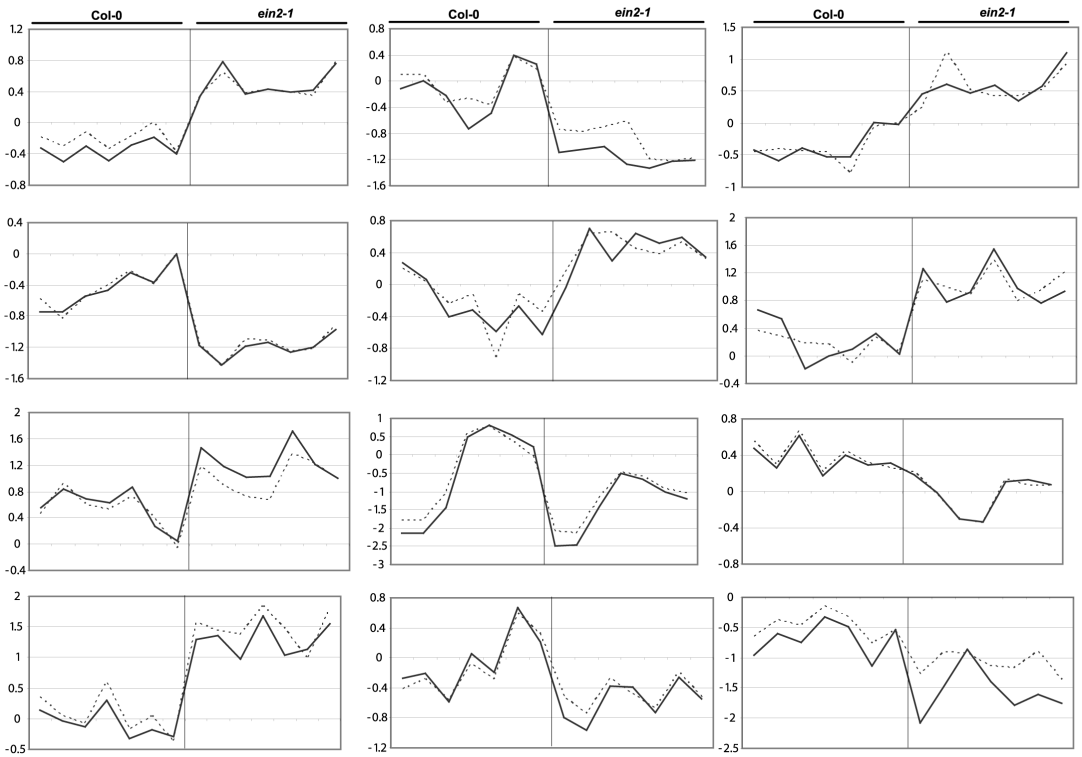


Figure 3: Reproducibility of the hybridisations: validation by comparing the hybridisation results of the twelve genes for which two independent cDNA clones were spotted on the array. In order of appearance from left to right and from top to bottom: At1g11580, At1g19350, At2g31390, At2g40940, At2g43510, At2g46750, At3g29030, At4g26080, At5g13650, At5g26260, At5g27320, At5g39190.

4.3.2 Cluster analysis

To group genes with similar expression patterns, the profiles of all 214 significantly differentially expressed genes were visualized using the hierarchical average linkage clustering (Eisen et al., 1998). Two main expression profiles could be identified: up-regulated genes (Fig 4A (A, B, and C)) and down-regulated genes (Fig 4A (D)). The genes in cluster A displayed a peak in expression at the latest time-point of treatment (6h), therefore these genes belong to the group of the later ethylene-induced genes. Within

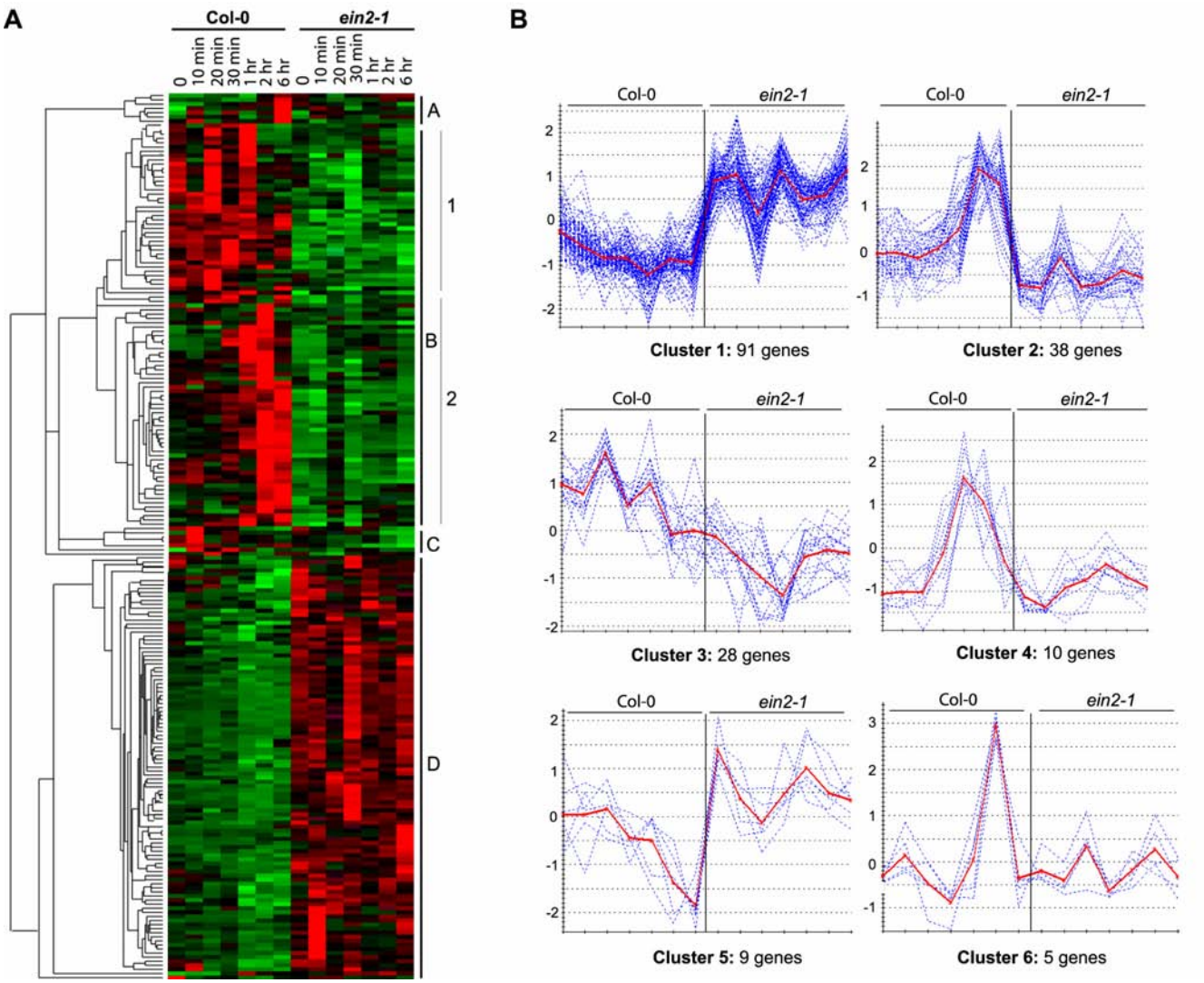


Figure 4: A. Hierarchical average linkage clustering result of the 214 ethylene-modulated genes out of the microarray analysis. Each column represents the time point of sampling during the treatment with 10ppm ethylene in both wild type and the *ein2-1* mutant and each row represents the expression profile of a transcript shown over the 7 time points for Col-0 and *ein2-1.* Red and green reflect transcriptional activation and repression respectively. Four main clusters are indicated on the left (A, B, C, D). Cluster B was divided in two subclusters (1, 2). B. Gene expression profiles obtained by adaptive quality-based clustering. The 214 differentially expressed genes were clustered using specific software (De Smet *et al*., 2002). Dashed lines represent the normalized expression profiles of the genes. The full line graphs represent the mean expression profile of all transcripts belonging to the respective cluster. Values on the y axis are the normalized expression data. Values on the x axis represent the 7 time points (0 to 6hr) for Col-0 (right panel) and *ein2-1* (left panel). For each cluster the number of genes is indicated.

cluster B, two subclusters could be identified based on the time points of highest expression level in the wild type. Subcluster B1 contains ethylene up-regulated genes during the earliest time periods of treatment and genes displaying a higher expression level during the earliest time points in wild type compared to *ein2-1*. For the genes belonging to subcluster B2 the expression level in wild type was significantly up-regulated by ethylene in wild type at later time points of treatment. From the top to the bottom of this cluster, a small shift from earlier to later ethylene regulation is visible. Cluster C is the

smallest cluster, containing genes that display a very early temporal increase in expression, much comparable to cluster A of the cDNA-AFLP. Finally, the biggest cluster, cluster D, contained down-regulated genes and genes with a higher basic level of expression in the insensitive mutant. Gene identity and extra information are provided as additional data (see 4.9-table 1 in this chapter).

In parallel to the Eisen clustering, the adaptive quality-based clustering (AQBC) algorithm was applied (De Smet *et al.*, 2002). Application of several clustering methods is not only useful as an independent confirmation of the expression profiles, but also allows discovering new interesting features. For this analysis, a probability of 0.95 was used and the minimal number of genes in a cluster was set to 5. The algorithm allowed clustering 181 out of the 214 genes into six clusters (Fig. 4B). Cluster 1 (91 genes) and cluster 2 (38 genes) were predominant and include transcripts repressed and induced by ethylene respectively. Cluster 3 (28 genes) corresponds largely to cluster B1 in the Eisen clustering. In addition to the previous clusters, the adaptive quality-based clustering method also presented three smaller clusters. Cluster 4 (10 genes) and cluster 6 (5 genes) contain transcripts that are specifically up-regulated after 1h-2h and 2h of treatment respectively. The genes belonging to cluster 5 (9 genes) are strongly repressed by ethylene, especially after 2-6h. Gene identity and extra information are provided as additional data (see 4.9-table 2 in this chapter).

4.3.3 Functional analysis

All differentially expressed genes were classified according to their functional categories derived from MatDB (http:/mips.gsf.de/). Figure 5 shows a graphical representation of the subdivision of the 214 differentially expressed genes into functional classes. The major functional groups are genes involved in metabolism; cell rescue, defence and virulence; subcellular localization, and genes involved in protein binding and cofactor requirement.

For the 3 largest clusters derived from AQBC-clustering (cluster 1, 2, 3), we could distinguish an overrepresentation of specific functional groups. To evaluate the importance of the predominant functional classes in a given cluster, the percentage of the genes belonging to the defined functional group was compared to that of all genes present on the array. In cluster 1 (Fig.6A), representing the main group of down-regulated genes, genes involved in cell rescue, defence and virulence, as well as metabolic genes and genes encoding proteins that facilitate transport are clearly overrepresented. In cluster 2 (Fig. 6B)

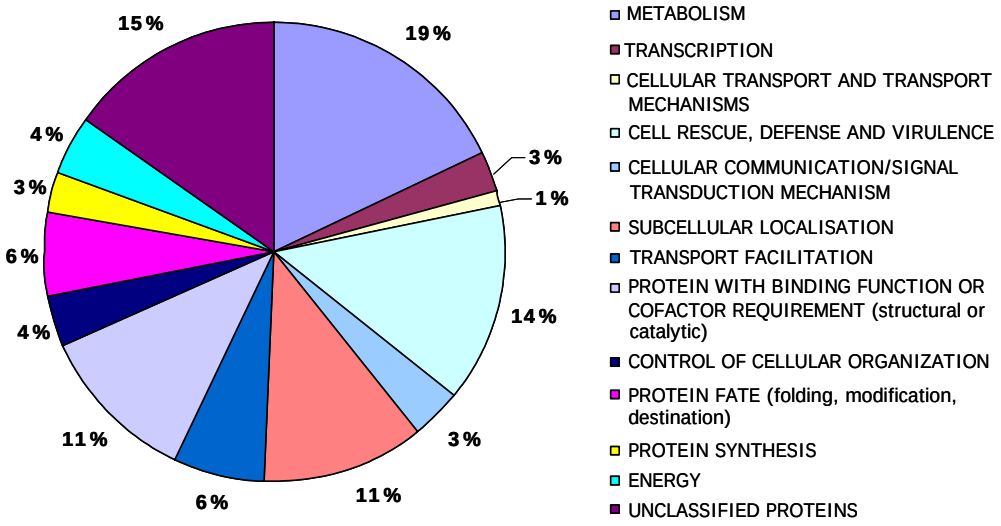


Figure 5: Pie-diagram representing the subdivision of the 214 differentially expressed genes into functional classes. The percentage of genes belonging to each functional class is indicated.

(up-regulated genes) there is again a clear presence of metabolic and defence genes, albeit to a lesser extent as in cluster 1 for the defence genes. These observations indicate that ethylene negatively regulates some metabolic processes while other metabolic processes are induced in the presence of ethylene. In addition, ethylene mainly decreases the transcription of defence genes but also induces some of these genes. In contrast, in cluster 3 (Fig. 6C); containing genes that are early modulated by ethylene, other functional classes are overrepresented apart from the metabolic genes. Early ethylene regulated genes particularly include genes needed for protein synthesis and protein activity. Moreover, genes involved in the control of cellular organization are also activated very early by ethylene. Altogether, it is clear that different classes of genes are regulated by ethylene at early compared to later time points.

Recently, a myriad of microarray data became publicly available from the GARNET facility at Nottingham University, UK (http://www.york.ac.uk/res/garnet/projects.htm), based on hybridization of Affymetrix chips covering 22.746 *Arabidopsis* genes. Expression data for different tissues were automatically analyzed using a specially designed algorithm (D. Zadik and M.Bennett, unpublished). To our surprise, about 25% of all ethylene-modulated genes are highly expressed in roots (Fig. 7). The second main group of genes (about 25%) is ubiquitously expressed whereas small groups of genes are more present in specific tissues.

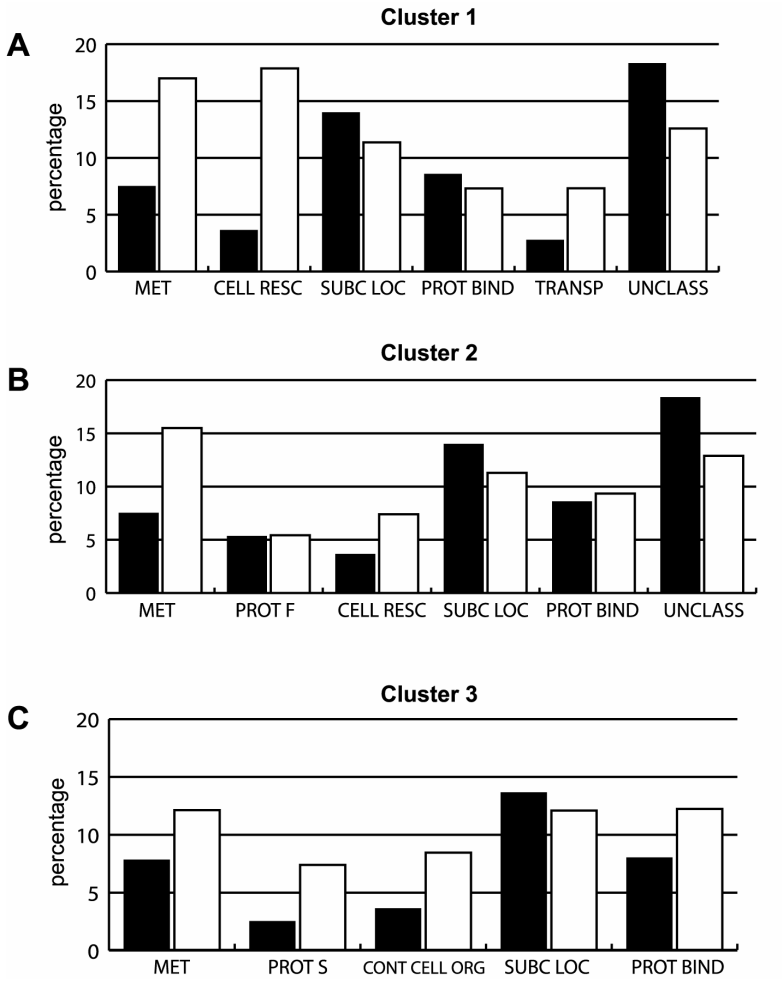


Figure 6: Graphical representation of the percentage of genes belonging to a given functional class for the major AQBC clusters. The three largest clusters are presented here (A, B, C). This percentage (white bars) was compared to the percentage of all genes present on the array belonging to the corresponding functional class (black bars). Y-axis: percentage; MET, metabolism; CELL RESC, cell rescue and defense and virulence; SUBC LOC, subcellular localization; PROT BIND, protein with binding function or cofactor requirement, TRANSP, transport facilitation; UNCLASS, unclassified proteins/classification not yet clear-cut; PROT F, protein fate (folding, modification, destination); PROT S, protein synthesis; CONT CELL ORG, control of cellular organization.

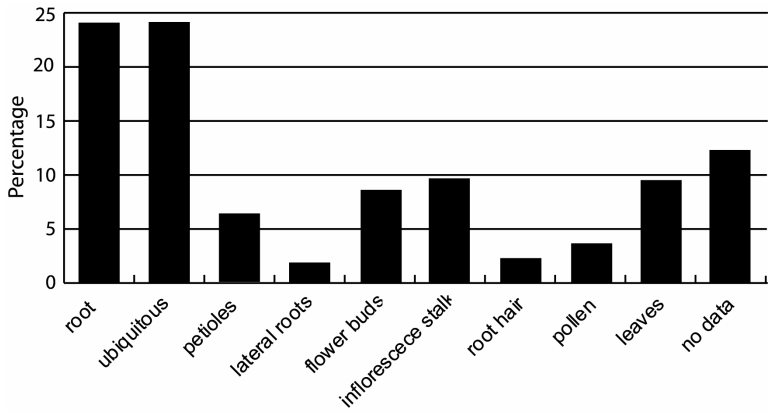


Figure 7: The graph represents the distribution of all ethylene-modulated genes in the different tissues based on Affymetrix (22.7K) expression data.

4.4 DISCUSSION

In this study, the transcriptional changes upon short ethylene treatment were studied using an *Arabidopsis* microarray. Of the 6008 genes present on the chip, 214 genes displayed a significantly ethylene-regulated pattern. Fifty-one was down-regulated by ethylene, 34% displayed a -mostly transient- up-regulated pattern and 15% was early modulated by ethylene. From the clustering and functional analysis of these differentially expressed genes, some major functional groups appeared. The most interesting observations are discussed below.

4.4.1 Ethylene in plant disease resistance and abiotic stresses

Besides its physiological role at different developmental stages, ethylene is also a stress hormone. Its synthesis is induced by a variety of stress signals, such as mechanical wounding, chemicals and metals, drought, extreme temperatures, and pathogen infection (Johnson and Ecker, 1998; Kende, 1993). However, depending on the plant species and the type of pathogen and its offensive strategies, the role of ethylene can be very different. Ethylene-insensitive signalling mutants may show either increased susceptibility or increased resistance (Bent et al., 1992; Thomma et al., 1999; Asai et al., 2000; Greenberg et al., 2000; Berrocal-Lobo et al., 2002). This apparent discrepancy among the roles of ethylene in different plant-pathogen interactions may be reconciled by the different infection mechanisms of different pathogens, and by the fact that ethylene is not only involved in pathogen response, but is also in many aspects of plant development including senescence, cell death, and ripening (Abeles et al., 1992). Therefore, the detrimental effect of ethylene in some plant-pathogen interactions may be an indirect consequence of the involvement of the hormone in the above-mentioned developmental processes. Besides its involvement in pathogen infection, ethylene is also implicated in response to abiotic stresses. An enhanced ethylene emanation is one of the earliest responses to ozone stress (Moeder et al., 2002; Vahala et al., 2003). It is suggested that ethylene is involved in the regulation of cell death by amplifying ROS production, which is responsible for the execution of spreading cell death (Overmyer et al., 2000). Compared with the two other hormones involved in responses to abiotic stress and pathogen defence, JA and SA, ethylene is involved in the early responses whereas JA and SA may control more prolonged effects. In agreement with these observations, a large group of genes involved in defence and disease were rapidly affected by ethylene in our analysis. Interestingly, these defence genes were mainly down-regulated by ethylene (see fig. 6) which is

contrary to what is described for the defence gene *PDF1-2*, and the PR-genes *PR-3* and *PR-4* in *Arabidopsis* (Penninckx et al., 1996). This observation is probably due to the emphasis on early time points whereas other studies focused on longer treatments with ethylene. In accordance with our findings, the defence gene catalase was down-regulated in tomato after 15 minutes of ethylene treatment (Zegzouti et al., 1999).

The heat shock protein 101, a superoxide dismutase, a catalase3-homolog, an HSR201-like, two putative disease resistant response genes, an osmotin precursor, a beta-glucanase precursor and a putative endochitinase were down-regulated by ethylene or displayed a lower level in the wild type compared to *ein2-1*. The HSR201 (hypersensitivity-related)-like protein was isolated during an incompatible interaction between tobacco and the bacterial pathogen *Pseudomonas solanacearum* (Czernic et al., 1996). Interestingly, *hsr201* is homologous to an ethylene-inducible tomato gene involved in fruit ripening and senescence. All major HSPs have related functions, protecting proteins against misfolding and aggregation. HSP101 seems to have a general role in thermotolerance, whereas for cpHsc70-1 a specific role in the chloroplast is hypothesized (Queitsch et al., 2000; Sung et al., 2001). The expression of the *HSP101* gene was down-regulated very early on and the amount of *cpHsc70-1* mRNA was higher in wild type compared to *ein2-1*.

One striking result is that a fair number of peroxidase genes are down-regulated by ethylene. All these peroxidases belong to the classical class III family of peroxidases which are targeted via the endoplasmic reticulum (ER) either extracellullarly or to the vacuole. They are ascribed a variety of functional roles, including lignification, suberization, auxin metabolism, defence, stress and developmentally related processes (Welinder et al., 2002). Many of these peroxidases were down-regulated when the organ was subjected to stress. Together with the other down-regulated defence genes, these peroxidases can be involved in the amplification of the cell death signal by loosening cell walls, inhibiting H_2O_2 scavenging, which leads to local resistance responses. Moreover, the mRNA for the phytocyanin blue copper binding protein is also down-regulated by ethylene and could have an analogous role since some phytocyanins are involved in redox reactions occurring during primary defence responses in plants and/or in lignin formation (Nersissian et al., 1998). A similar pattern was observed for the mRNA of thioredoxin H-type 3 (*TRX-H-3*). It was demonstrated that TRX-H-3 induces H_2O_2 tolerance in Saccharomyces cerevisiae, suggesting that these isoforms could act as antioxidants possibly by serving as hydrogen donors for a thioredoxin-dependent peroxidases (Verdoucq et al., 1999; Brehelin et al., 2000). Recently, a new antioxidant route is described for the action of the enzyme methionine sulfoxide reductase (Nakao et al., 2003). In our study, the methionine sulfoxide

reductase (SelR) domain protein also displays a down-regulated pattern. Finally, a superoxide dismutase, known as an antioxidant, was down-regulated, whereas a Cu/Zn-superoxide dismutase copper chaperone precursor (*CCS*) and a Cu/Zn superoxide dismutase were early induced by ethylene.

Another group of ethylene-regulated genes encode enzymes in intermediate steps in lignin biosynthesis. These include two cinnamoyl-coA reductase-like genes and the cinnamyl-alcohol dehydrogenase *ELI3-1*. For both enzymes other isozymes have been reported to function in defence and wounding response (Kiedrowski et al., 1992; Cheong et al., 2002). One of the two cinnamyl-coA reductase-like genes and the *ELI3-1* gene are both up-regulated by ethylene at the latest time points of treatment (2-6h), suggesting a more protective role for ethylene at later stages of defence. The other cinnamyl-coA reductase-like gene is down-regulated by ethylene. Two other genes involved in lignin synthesis in plants are a putative laccase, with an action similar to that of the plastocyanin blue-copper containing protein (Claus, 2003) and a 4-coumarate-CoA ligase-like protein, similar to a key enzyme of phenylpropanoid metabolism (Lee et al., 1995).

Interestingly, *RSH3* (ReIA/SpotT homolog) was induced by ethylene after 1hr of treatment. ReIA and spotT play a central role in the bacterial stringent response. These enzymes both transfer pyrophosphate groups from ATP to the 3' positions of GDP and GTP, resulting in the rapid accumulation of ppGpp and pppGpp, which induces and represses transcription of genes involved in different processes, including response to biotic and abiotic stress (van der Biezen et al., 2000). This observation is a first indication for a possible ethylene regulation of ppGpp and pppGpp as intracellular secondary signalling molecules.

A number of transcription factors –amongst which the WRKY DNA-binding proteins- are associated with plant defence responses. Dong et al. (2003) demonstrated that WRKY transcription factor 11 is induced within the first hours after treatment with an avirulent pathogen or with SA (Dong et al., 2003). *WRKY11* showed little change in transcript level after treatment with ethylene, but the amount of mRNA was about 2.5 fold higher in *ein2-1* compared to the wild type, indicating that the involvement of ethylene cannot be ruled out.

Another group of early-downregulated defence-related genes are three putative myrosinase-binding proteins, two putative lectins, and a putative jasmonate-induced protein of the jacalin lectin family. Jacalin-related proteins have been suggested to be involved in resistance against bacteria, insects and fungi (Chisolm et al., 2000). Myrosinase is an enzyme capable of hydrolyzing glucosinolates into various compounds that function in defence (Taipalensuu et al., 1996). In *B. napus MBPs* (myrosinase binding

protein genes) are up-regulated systemically in response to JA during insect or fungal attack. Down-regulation by ethylene is also found for the pleiotropic drug resistance gene *PDR9* gene belonging to the ATP-binding cassette (ABC) transporters. These proteins have been implicated in the transport of antifungal agents (van den Brule and Smart, 2002). In contrast, the mRNA of an ABC transporter-like gene (At5g06530) displayed a peak in expression after 1-2h of treatment and a putative ATP-dependent transmembrane transporter was up-regulated by ethylene. Their role can be diverse since ABC-transporters seem to play a central role in plant growth and developmental processes (Martinoia et al., 2002).

Two putative trypsin inhibitors displayed a down-regulated pattern. These defence-related-proteins are known to be involved in plant protection (Urwin et al., 1998; Vercauteren et al., 2001).

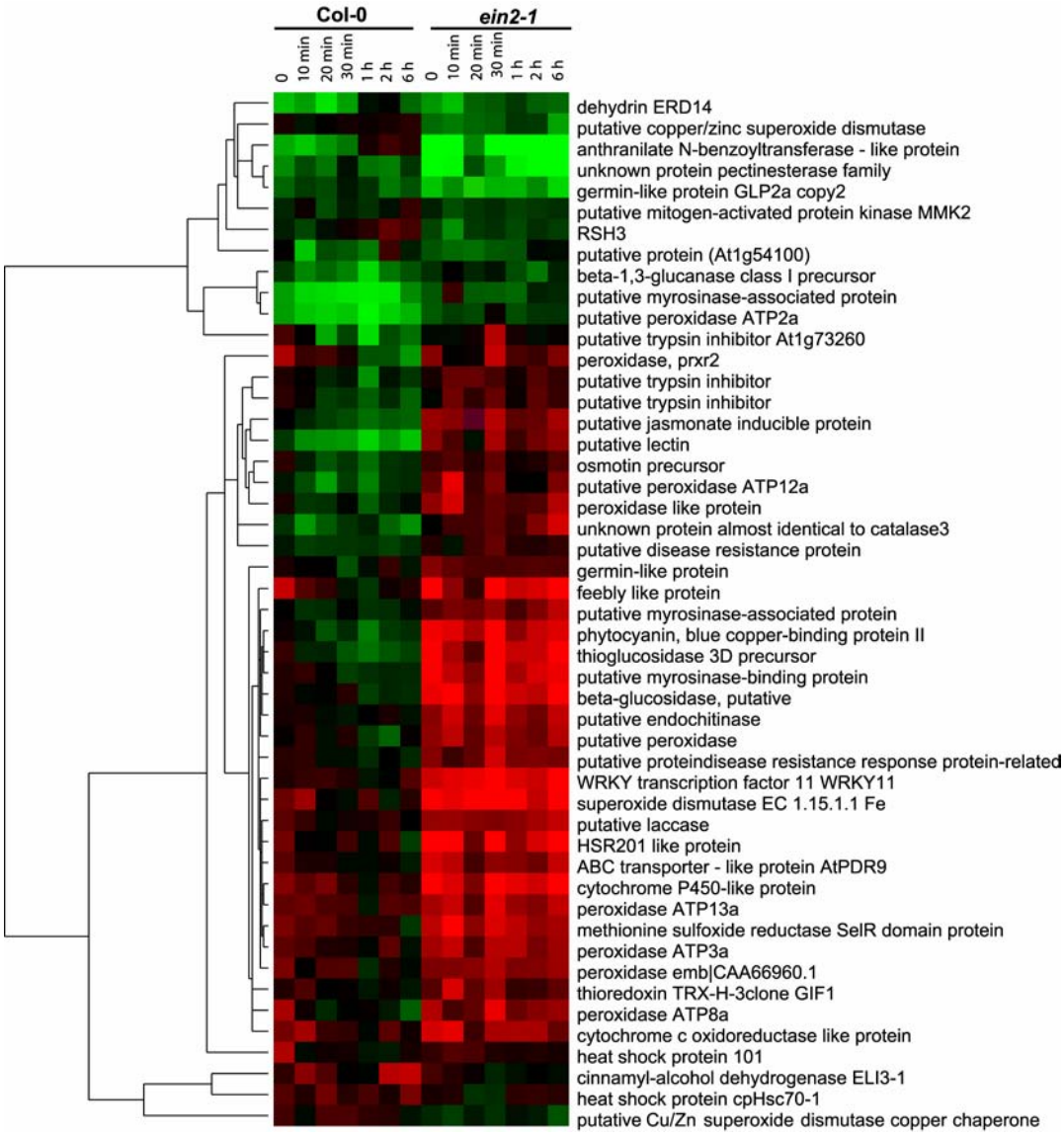


Figure 8: Hierarchical average linkage clustering result of all differentially expressed genes involved in defence, cell rescue, virulence, and detoxification. Each line, representing a differentially expressed gene, contains its description based on the MIPS protein database.

The mRNA that codes for the anthranilate N-benzoyltransferase-like protein was highly up-regulated after 1h of treatment. This enzyme plays a role in biosynthesis of phytoalexins which are important in plant defence against microorganisms (Yang et al., 1997).
Out of these observations, we can conclude that ethylene is probably an active component differentially controlling the damage process depending on the infection phase. Ethylene mainly inhibited several groups of enzymes involved in the early defence response to abiotic stresses and pathogens, although the expression of a smaller group of defence-genes was stimulated. The response to ethylene for the latter group of genes was mostly more specific to the later time points. This reaction can possibly switch on the JA and/or the SA signalling pathways which are involved in more prolonged effects in defence and resistance.

4.4.2 Cell wall metabolism

Expansins are cell-wall-loosening proteins that induce stress relaxation and extension of plant cell walls. In this analysis two α-expansins (EXP) were found to be regulated by ethylene. For *EXP5* down-regulation was observed after 2 hours of treatment. In contrast. the putative *EXP11* displayed a rapid 2-fold transient peak in expression (10min). This observation strengthens the hypothesis that at early time-points ethylene plays a role in cell-wall loosening (cf. defence-responses) while at later time-points it appears to have a protective role enforcing the cell wall (cf. genes involved in lignin biosynthesis). Cosgrove et al. (2002) suggested a wall structural model in which cellulose microfibrils are linked together by a relatively large and inaccessible xyloglucan complex that is opened by EXP, which allows immediate wall extension and access to enzymatic attack by endoglucanases. (Cosgrove et al., 2002). Thus, the xyloglucan endo-1,4-beta-D-glucanase-like protein could be involved in a cooperation with EXP11 since the former is upregulated after 20 minutes of treatment and reaches its highest level after 1 hr, subsequently dropping below control level. The arabinogalactan-protein gene *AGP1* displays a similar pattern of expression. AGPs are hydroxyproline-rich cell wall proteins implicated in plant growth and development. In previous research *AGP1* was shown to be affected by different forms of stress (Schultz et al., 2002). Moreover, in the AFLP-based TP-analysis, *AGP7* displayed a similar expression pattern (see Chapter 2, AQBC-cluster 5, p 55). Finally, a putative pectin methylesterase was down-regulated by ethylene.

4.4.3 Novel interactions between responses to ethylene, ABA, sugar, and auxin

Genetic analysis of ethylene and ABA suggested that these hormones antagonize each other at the level of germination. After germination, ABA and ethylene signalling display complex interactions. Since both hormones inhibit root growth, they may act in the same or in parallel pathways. However, ethylene-overproducing mutants have decreased ABA sensitivity, implying another antagonistic interaction. One suggested explanation for this apparent inconsistency is that ABA inhibits root growth by signalling through the ethylene response pathway, but is unable to use this pathway in the presence of ethylene (Ghassemian et al., 2000). Furthermore, both hormones do not appear to interact in ABA-regulated processes such as stomatal closure or induction of some ABA-response genes. In our analysis, both *ABI1* and *ABI2* were highly up-regulated by ethylene with a peak of expression after 1-2h of treatment. Both genes encode homologous type 2C protein phosphatases acting as negative regulators in ABA signalling (Gosti et al., 1999). Since it is known that *ABI1* is up-regulated by ABA (Leung et al., 1997), we can hypothesize that both hormones work additively in the regulation of transcription of these genes. Up-regulation by ethylene was also observed for the abscisic acid responsive elements-binding factor *(ABRE/ABF3*), which is an ABA-inducible bZIP transcription factor, and for dehydrin *ERD14*. The pattern of ABA-inducibility of *ABF3* (Choi et al., 2000) corresponds to that seen by ethylene-treatment. Moreover, this transcription factor was also induced by high salt treatment. The protein level of ERD14 was also up-regulated by salinity and low temperature (Nylander et al., 2001). In addition, nitrate reductase 1 (*NR1*) displayed a peak in expression after 1h of treatment. NR is the first enzyme of the nitrate assimilation in plants and hormonal control of the transcription and activity of NR1 is described Moreover, NR-mediated NO synthesis in guard cells is required for ABA-induced stomatal closure (Desikan et al., 2002). An integration between ABA and ethylene in this response is possible. Although the interaction at this stage of development is not yet clear, it can be concluded that for some responses both hormones work in parallel on the expression of genes involved in ABA-signalling. Another possibility is that the ethylene signal could be mediated by ABA. However, this is contradictory to what is known for *era3*, which is allelic to *ein2* and overaccumulates ABA (Ghassemian et al., 2000). In view of these data, the *ABI1/2* and the *ABF3* genes (which are ABA-inducible), should display a higher expression level in *ein2*. Therefore, as suggested by Ghassemain et al (2000), ABA could use the ethylene response pathway, but only in the absence of ethylene.

Characterization of sugar-signalling mutants in *Arabidopsis* has unraveled a complex

signalling network that links sugar responses to ABA and ethylene (Zhou et al., 1998; Leon and Sheen, 2003). Glucose activates ABA biosynthesis, and ABA, glucose, and ethylene signalling antagonize each other (Leon and Sheen, 2003). In this perspective the down-regulation of expression of a putative hexose transporter and a sugar transporter-like gene, provides a new view on the antagonistic relationship between sugar and ethylene signalling. Alternatively, ethylene regulation of sugar transporters could also result in delayed senescence and increased stress resistance since sugars are known to repress photosynthetic gene expression (Leon and Sheen, 2003). Moreover, a putative fructokinase displayed a lower level in expression in the wild type compared to *ein2-1,* indicating that ethylene is also negatively involved in sucrose metabolism.

Interactions between ethylene and auxin pathways were also observed in this study. One striking result is the induction of UDP-glucose:indole-3-acetate beta-D-glucosyltransferase (iaglu) upon prolonged ethylene exposure (1 up to 6h). IAA has been shown to form conjugates with sugars, amino acids, and small peptides, which are believed to be involved in IAA transport, in IAA storage, in the homeostatic control of the pool of free hormone, and as a first step in catabolic pathways (Jackson et al., 2001). To our knowledge, this is the first report showing the involvement of ethylene in IAA conjugation. In addition, one of the primary auxin response genes, *IAA3* (MIPS description: putative

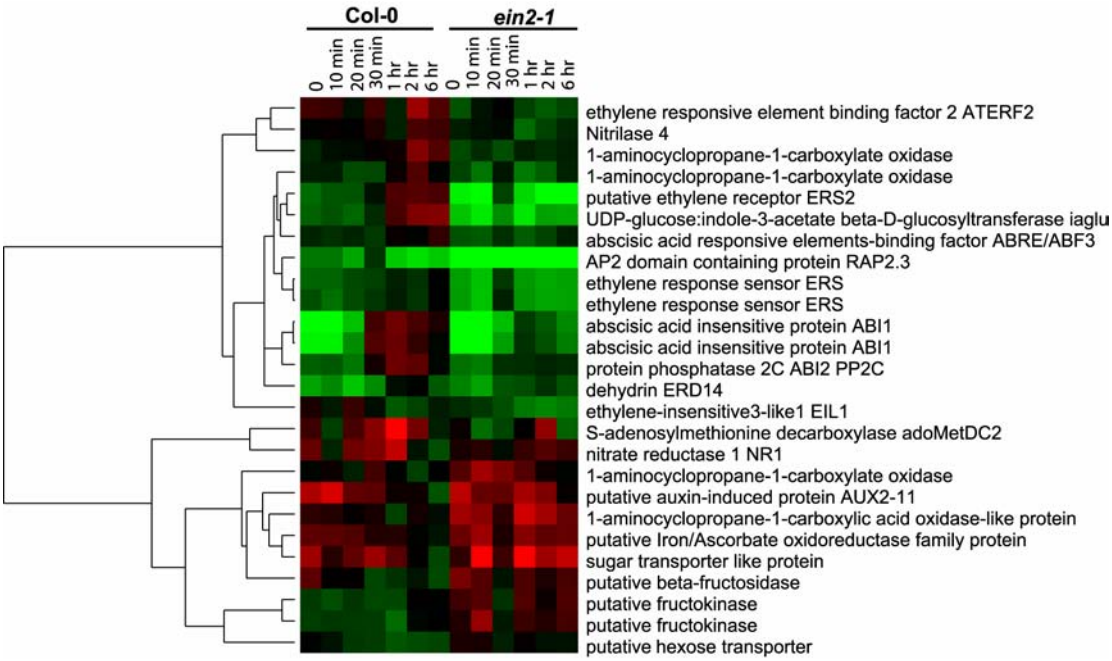


Figure 9: Hierarchical average linkage clustering result of all differentially expressed genes involved in the interaction ethylene-ABA/auxin/sugar signalling and the autoregulation of ethylene biosynthesis, signalling and related components. Each line, representing a differentially expressed gene, contains its description based on the MIPS protein database.

auxin-induced protein AUX2-11), is significantly down-regulated by ethylene from 1h treatment on. IAA3 plays a central role in auxin signalling as a repressor of auxin-regulated gene expression and may also regulate light responses (Tian et al., 2002).

4.4.4 Autoregulation of ethylene biosynthesis, signalling and related components

Several genes encoding components of the ethylene biosynthetic and signalling pathway displayed an ethylene-regulated pattern. For some of these genes, ethylene-inducibility was already known, corroborating our results. As for ethylene biosynthesis, three ACC-oxidases (ACO) and one ACC-oxidase-like gene were affected by ethylene. ACO is present as a multigene family in *Arabidopsis*, but little information about these genes has been reported. In other plant species, it has been shown that ACO genes are differentially expressed during development and upon different treatments (Lasserre et al., 1996). In our analysis differential expression of the four ACOs was observed. The expression of two of them (At1g05010 and At5g43450) peaked after 2 hours of treatment, whereas the gene encoding an ACC-oxidase like protein was down-regulated in wild type, and the fourth ACO (At2g19590) only displayed a higher level in the *ein2-1* mutant. Ethylene-regulated ethylene biosynthesis implicates ethylene as a messenger for the induction of later ethylene-responsive genes or amplification of the ethylene signal. Moreover, the differential expression of multiple ACOs proves a more complex autoregulation of ethylene biosynthesis.

The putative Iron/Ascorbate oxidoreductase family protein is structurally similar to the tomato ethylene synthesis regulatory protein E8. E8 is a fruit ripening protein related to ACO and has a negative effect on ethylene production in fruit (Penarrubia et al., 1992). In our analysis, this gene showed a clear down-regulation by ethylene during the 6 hours time-course.

S-adenosylmethionine decarboxylase (*adoMetDC2*), displaying a transient peak in expression after 1hr of treatment, is a key enzyme in the biosynthesis of the polyamines spermine and spermidine (Franceschetti et al., 2001). Since the ethylene and polyamine biosynthetic pathways are linked through S-adenosylmethionine, (which is then converted to ACC by ACC synthase), the up-regulation of *adoMetDC2* by ethylene may allow a negative control on its own biosynthesis. An alternative explanation for the induction of adoMetDC2 by ethylene can be related to the protective role since polyamines have been shown to play a role in stress response. Moreover, arginine carboxylase, involved in the first steps of polyamine synthesis, also displayed a peak in expression after 20 minutes of

treatment, indicating that ethylene stimulates this pathway at different levels.

Interestingly, nitrilase 4 (*NIT4*) expression is up-regulated by ethylene. This gene encodes a beta-cyano-1-alanine hydratase/nitrilase and is proposed to take part in cyanide detoxification (Piotrowski et al., 2001). NIT4 activity was higher in senescent *Arabidopsis* leaves. Therefore, NIT4 may play a role in cyanide detoxification during ethylene biosynthesis.

Besides ethylene-regulated transcriptional control for genes in ethylene biosynthesis, also genes encoding factors involved in ethylene signalling were affected. This was the case for *ERS1*, *ERS2*, *AtERF2* and *EIL1*. The induction by ethylene for the receptors *ERS1* and *ERS2* and the transcription factor *AtERF2* was reported before (Hua et al., 1998; Fujimoto et al., 2000). *EIL1* mRNA displayed a higher level in expression in wild type compared to *ein2-1*. The importance of EIL1 for sensitivity to ethylene throughout the life cycle was recently shown by Alonso et al. (2003). In *Arabidopsis* no prior expression studies of EIL1 are reported, whereas in carnation flower tissues the amount of DC-EIL1 decreased by exogenously applied ethylene (Waki et al., 2001). A similar pattern of ethylene-stimulated expression was observed for the AP2 domain containing protein gene *RAP2.3*. This proteins belongs to the EREBP-like class of RAP2 (related to AP2) transcription factors (Okamuro et al., 1997). Similar AP2 gene families have been reported to be induced by SA, JA, ethylene, and pathogen attack (Maleck et al., 2000; Schenk et al., 2000).

4.4.5 Transcriptional and post-transcriptional regulation

Ethylene treatment causes up-or down regulation of genes involved in several steps starting from regulation of transcription of until peptide/protein synthesis. At least 11 genes encoding DNA-binding/transcription factors showed significant differences in expression by ethylene treatment. These include *AtERF2*, *EIL1*, AP2 domain containing protein *RAP2.3, ABF3*, *IAA3*, *WRKY11* (previously discussed), a nucleoid DNA-binding-like, a putative DNA binding, DNA-binding GT-1, and two zinc finger protein-like proteins. The putative DNA-binding protein gene, *GT-1*, is down-regulated by ethylene. GT-1 may be involved in the regulation of transcription of many different genes (Le Gourrierec et al., 1999). Since this gene is transcribed ubiquitously, it is proposed that transcriptional regulation by GT-1 may be achieved by interaction with other specific factors or by post-transcriptional modification. Thus, ethylene could be a regulator of this transcription factor. Furthermore, some components of the translational machinery are regulated by ethylene at the transcriptional level. These include the ribosomal protein genes L36-like, L30, ribosomal

protein S7, a glycine-rich RNA binding protein, a putative RNA binding protein gene (*RBP47*) (all up-regulated at later time-points) and the translation initiation factors 4E, IF2, a putative elongation factor G, an unknown protein similar to elongation factor TS, a putative splicing regulator protein, and a putative mRNA capping enzyme. This last group of genes mainly displayed a higher expression level during the early time points in wild type compared to *ein2-1* (see AQBC-cluster 3). The eukaryotic translation initiation factor 4E displayed a transient down-regulated pattern. This gene is one of the cap binding protein isoforms that interacts with the potyviral genome-linked protein Vpg and therefore may play a role in translational initiation, genome stability or intracellular trafficking of the viral RNA (Lellis et al., 2002).

Ethylene also regulates proteolytic degradation as seen for a putative cysteine proteinase, a subtilisin proteinase and a putative protease SppA. The putative cysteine proteinase was transiently 2-fold up-regulated after 10 minutes of treatment. A similar profile was seen for the cysteine proteinase gene *RD21A* in our previously described TP-experiment (see Chapter 2, AQBC-cluster A, p 55).

4.5 GENERAL CONCLUSION

Our study confirmed the importance of ethylene in both abiotic and biotic stresses and provided new insights into the earlier described role of ethylene in these processes. Furthermore, the results reveal novel understanding of the integration of ethylene signalling and two other hormones, ABA and auxin; and between ethylene and sugar signalling. Future characterization of the genes will help to better understand early responses to ethylene.

4.6 COMPARISON AND EVALUATION OF CDNA-AFLP VERSUS MICROARRAYS

Because both a hybridization-based and a PCR-based approach with a similar setup were used in this project, we were able to compare and evaluate both methods. With the microarray technique about one fourth of the *Arabidopsis* transcriptome was investigated. From this analysis, we identified 214 ethylene-modulated genes, representing 3.3% of all genes present on the chip. On the other hand, only ± 5% of the transcriptome was scanned by cDNA-AFLP. In total, about 1200 transcript tags were visualized and ± 4% of these were responsive to ethylene. Thus, both analyses yielded a similar percentage of differentially regulated genes. The small difference can be attributed to the presence of the

ctr1-1-specific genes in the cDNA-AFLP (the *ctr1-1* mutant was not investigated in the microarray analysis) or due to the different statistical approaches used in both analyses.

Since in both cases no full genome-wide transcription analysis was performed, a strict comparison of the methods is limited to the overlapping genes. When comparing the data set from the cDNA-AFLP experiment with all genes present on the 6K chip, only 15 genes of the 47 isolated genes are also present on the chip. From these 15 genes, 8 display a similar pattern in both analyses. This means that the pattern of 7 genes does not correspond between the two methods. Different arguments for these differences can be raised. First of all, in the cDNA-AFLP experiment the *ctr1-1* mutant was added. So, those genes being highly up- or down-regulated in the *ctr1-1* mutant (see cluster B and E in Chapter 2 on cDNA-AFLP, pages 55-56) can be present on the chip, but the pattern cannot be confirmed because no results for the *ctr1-1* mutant are available from the microarray experiment. This is the case for 3 of the 7 genes for which the pattern did not correspond between both methods. Another reason for the conflicting results could be that these genes are erroneously isolated due to technical errors in the procedure of the cDNA-AFLP. However, we have to keep in mind that one of the major disadvantages of the microarray includes the cross-hybridization. Therefore the compared data could be of two similar, but not identical genes. Another fact to consider is that a small percentage of the genes on the microarray are known to be misannotated. If this is the case for one of more of this small group of genes, then again two different genes were compared.

In addition to the technical differences between the two methods, they also differ at another level. On the microarray, Unigene clones from Incyte are spotted, which cover ESTs mainly originated from leaves. Therefore, we can expect that most of these clones belong to the class of abundant mRNAs. On the other hand, by using three selective bases in the cDNA-AFLP, the tags on the gels are covering mRNAs belonging to the less abundant and rare class of transcripts. Nowadays, full genome chips are available and therefore more suitable to compare with the cDNA-AFLP method, although the sensitivity when using microarrays is still a limitation compared to cDNA-AFLP.

Nevertheless, when looking at the functional groups of ethylene-regulated genes, a clear overlap between both transcript profiling methods can be seen. Both analyses indicated a role for ethylene in defence, transcriptional- and post-transcriptional regulation, cell wall metabolism, hormone interplay, signal transduction and protein degradation. Transcripts picked up in both analyses belonged to similar functional classes.

A significant overlap was observed between genes involved in cell wall metabolism: arabinogalactan proteins, pectine esterases, and xyloglucan-endotransglycolase/endoglucanases.

In addition, the defence-related gene Cu/Zn-superoxide dismutase copper chaperone precursor was early up-regulated in both approaches. Besides these, also heat shock proteins were found in both analyses.

The transcript level of two different cysteine proteinases was very early increased and other genes involved in protein degradation appeared in both analyses. The protease inhibitor Dr4 was down-regulated in both approaches, while two trypsin inhibitors also displayed a down-regulated expression pattern in the microarray analysis. Moreover, a subtilisin proteinase and a putative protease SppA were up-regulated by ethylene in the microarray analysis. Altogether, these results indicate a positive role for ethylene in protein degradation. At first sight, the involvement of ethylene in the ubiquitination pathway was not really confirmed by the microarray results; only one unknown protein is similar to the ubiquitin-specific protease 12. However, when taking a closer look at the data, several components of the ubiquitination pathway (UBC-like, ubiquitin carboxyl-terminal hydrolase) were responsive to ethylene but did not end up in our final data set because the induction-fold was just below two.

Concerning transcriptional and post-transcriptional regulation myb- and bHLH-related transcription factors, and also posttranscriptional initiation and elongation proteins appeared in both datasets. This was also true for the G-proteins and other signalling components. Finally, the regulation of ethylene on its own synthesis and on the auxin signalling pathway was reproduced in both systems.

Altogether, both transcript profiling methods revealed striking similarities in the genes triggered upon short term exposure to ethylene.

4.7 ACKNOWLEDGEMENTS

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A modified version of this chapter (together with chapter 4) is submitted to *the Plant Journal*.

4.8 EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. (ecotype Columbia-0) were purchased from Lehle seeds (Round Rock, TX). Ethylene response mutants *ctr1-1* and *ein2-1*, both in Col-0 background, were obtained from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University. Seeds were sown under sterile conditions as described previously by (Smalle et al., 1997). The growth medium used was MS/2 (half-strength Murashige and Skoog; Sigma supplemented with 1% sucrose). After sowing, plates were stored at 4°C in the dark for 1 day and then put in a growth chamber at 22 °C and 60% relative humidity under white fluorescent light (Photosynthetic photon flux density (PPFD): 75mmoles/m^2 s) and long day conditions (16 h light/ 8 h dark). Plants were 19 days old at the time of ethylene exposure.

Ethylene treatments

Plants were placed inside growth chambers dedicated to gas exposures. Control plants and the plants to be treated with ethylene were placed in two adjacent identical growth cabinets. Ambient conditions were 22 °C, 60% humidity and white fluorescence light (75mmoles/m^2 s) under long day conditions (16h light/ 8h dark). 10ppm of ethylene in air (organic carbon free, Air Liquide Belge N.V., Aalter, Belgium) were flushed through at a flux rate of 4 refreshments per hour. In order to allow identification of immediate and early response genes, harvesting was performed after 10 min., 20min., 30min., 1h, 2h, and 6h of treatment. Control samples treated with air were harvested after 10 min.

Microarray preparation

The microarray consisted of 6008 *Arabidopsis* genes composed from the unigene clone collection from Incyte (*Arabidopsis* Gem I, Incyte, USA) and 520 positive and negative controls (for details see www.microarray.be/service/currentlyavaillablearrays). All 6528

clones were spotted in duplicate on a single array. The cDNA inserts were PCR amplified using M13 primers, purified with Multiscreen-PCR plate (cat: MANU03050, Millipore, Belgium) and arrayed in 50% DMSO on Type VII silane coated slides (cat# RPK2331, Amersham Bioscience, Buckinghame, UK) using a Molecular Dynamics Generation III printer (Amersham BioSciences). Slides were blocked in 2xSSPE, 0.2%SDS for 30 minutes at 25°C.

Microarray hybridization

Total RNA was extracted from the samples using Trizol^{R} reagent (GIBCO/BRL, Gaithersburg, MD) according to the manufacturer's instructions.

A minimum of 5 μg total RNA was linearly amplified using in vitro transcription as described in detail in Puskás et al. (2002). The probes were resuspended in 210ml hybridization solution containing 50 % formamide, 1x HybridizationBuffer (cat# RPK0325, Amersham BioSciences, UK), 0.1 % SDS and 60μg/ml poly-dT. Hybridization and post-hybridization washing was performed at 45 ºC using an Automated Slide Processor, ASP (Amersham BioSciences, UK). Post-hybridization washing was performed in 1xSSC, 0.1% SDS, followed by 0.1xSSC, 0.1% SDS and 0.1xSSC. The complete ASP program can be downloaded from www.microarrays.be/technology/protocols .

Data analysis

Arrays were scanned at 532 nm and 635 nm using a Generation III scanner (Amersham BioSciences, UK). Image analysis was performed with ArrayVision (Imaging Research Inc, Ontario, Canada). A reference design was constructed, including reciprocal labeling of all samples. As a reference, we used a pool of *ctr1-1* samples (0-10min.-6hr. treated samples). Spot intensities were measured as artifact removed total intensities (ARVol) without correction for background. For 24 negative control spots containing a Bacillus subtilis specific cDNA and 6,008 *Arabidopsis* spots, we first addressed within-slide normalization by plotting for each single slide a "MA-plot" (Yang et al., 2002) where M = $\log_2$ (R/G) and A = $\log_2$ $\sqrt{RXG}$ for each spot. The "LOWESS" normalization (*f*=0.2) was applied to correct for dye intensity differences. Based on the M' (adjusted M) and A values for each gene, adjusted $\log_2$R and $\log_2$G signal intensities were obtained. Based on the 96 adjusted $\log_2$R and $\log_2$G signal intensities of the negative control spots, the median and the 95 Percentile were calculated. The 95 Percentile was arbitrarily defined as the

signal threshold. For each gene, the adjusted $\log_2$R and $\log_2$G signal intensities were compared to the signal threshold; 749 genes were below the signal threshold in at least 25% of the number of observations in the two test samples (n = 56) per gene and were subsequently removed from the data set. All values of the remaining 5259 genes below the 95 Percentile threshold were reset to the median value of the negative control intensities.

Mixed ANOVA models, in which some effects are considered fixed and others are considered random, have been used as follows (Wolfinger et al., 2001): 1) let y_{iklm} be the base-2 logarithm of the "Lowess"-transformed spot measurement from gene i (i = 1…5259); we first applied a linear normalization ANOVA model of the form $y_{iklm} = \mu + A_k + (ADR)_{klm} + \varepsilon_{iklm}$ to estimate global variation of the collection of i selected cDNA fragments in the form of random array effects (A_k; k = 1…28), random channel effects for the mth replication of the total collection of cDNA fragments ($(ADR)_{klm}$; k = 1…28, l = 1…2, m = 1…2) and random error ε_{iklm}. Residuals, computed by subtracting the fitted values for the effects from the y_{iklm}, were then subjected to 5,259 gene-specific models of the form $r_{ijgklmn} = \mu + (GD)_{il} + (GS)_{ij} + (GST)_{ijn} + (GSV)_{ijg} + (GSVT)_{ijgn} + (GA)_{ik} + \gamma_{ijgklmn}$ partitioning partitioning gene-specific variation into fixed gene-specific dye effects ($(GD)_{il}$; l = 1…2), fixed sample effects ($(GS)_{ij}$, j = 1… 2; control (ctr1-1) and test sample (Col-0 and *ein2-1*)), fixed time effects ($(GST)_{ijn}$, n = 1…7), fixed genotype effects ($(GSV)_{ijg}$, g = 1…2; Col-0 and *ein2-1*), fixed genotype-specific response in time effects (effect of interest) ($(GSVT)_{ijgn}$, g = 1…2, n = 1…7), random spot effects ($(GA)_{ik}$, k = 1…28) and random error $\gamma_{ijgklmn}$.

Assigning significance to the expression differences for the fixed main effects genotype and time and the interaction term genotype×time was based on the Wald statistic, calculated for each term and for each gene, and tested against the X^2-distribution. The cut-off selection for differentially expressed genes was based on two criteria. A first cut-off was set at p<0.001; 476, 1368 and 231 genes had a significant expression difference for the fixed effects genotype, time and genotype×time, respectively. Genes only differentially expressed over time were considered as affected by circadian rhythm or mechanical stress and were therefore ruled out for the further analysis. The second criterium was a more than two-fold difference in expression between the two genotypes *ein2-1* and Col-0. A group of 214 significantly ethylene regulated genes remained from this dual selection.

All standard calculations and statistics, including the LOWESS fit were done using Genstat (Genstat Release 6.1 for Windows, VSN International, Hemel Hempstead, UK). We used Genstat REML procedure (Payne and Arnold, 2000) to perform both the normalization and

gene model fits. The Genstat “VWALD” procedure (Goedhard and Thissen, www.biometris.nl/software/genstat) was used to save the non-hierarchical Wald test for the fixed terms in the REML analysis.

4.9 ADDITIONAL DATA

Table 1: Overview of ethylene-regulated genes from the microarray analysis according to the Eisen clustering

Description (MIPS)	Description (TIGR)	Accession	Cluster
ghypothetical protein	isochorismate synthase (isochorismate mutase), putative	At1g18870	cluster A
eukaryotic translation initiation factor 4E, putative	eukaryotic translation initiation factor 4E , putative	At1g29550	cluster A
unknown protein, 5' partial	expressed protein	At3g22105	cluster A
unknown protein	expressed protein	At3g14190	cluster A
unknown protein	expressed protein	At5g43970	cluster A
glycine-rich RNA binding protein, putative	RNA-binding protein, putative	At3g23830	cluster A
putative mitogen-activated protein kinase MMK2	mitogen-activated protein kinase (MAPK), putative (MPK2)	At1g59580	cluster A
nitrate reductase 1 (NR1)	nitrate reductase 1 (NR1)	At1g77760	cluster A
xyloglucan endo-1,4-beta-D-glucanase-like protein	xyloglucan endotransglycosylase, putative	At4g30280	cluster B1
arabinogalactan-protein AGP1	arabinogalactan-protein (AGP1)	At5g64310	cluster B1
calcium-dependent protein kinase	calcium-dependent protein kinase family (CDPK)	At5g66210	cluster B1
putative protein	expressed protein	At1g80865	cluster B1
S-adenosylmethionine decarboxylase (adoMetDC2)	adenosylmethionine decarboxylase family	At5g15950	cluster B1
plasma membrane proton ATPase-like	ATPase, plasma membrane-type (proton pump), putative	At5g62670	cluster B1
putative receptor-like protein kinase, ERECTA	leucine rich repeat protein kinase family (ERECTA)	At2g26330	cluster B1
unknown protein	elongation factor Ts family	At4g29060	cluster B1
putative SF16 protein {Helianthus annuus}	calmodulin-binding protein family	At2g43680	cluster B1
GTP-binding protein typA	GTP-binding protein typA	At5g13650	cluster B1
elongation factor G, putative	elongation factor Tu family protein	At1g62750	cluster B1
putative protein	WD-40 repeat protein family	At1g15750	cluster B1
putative translation initiation factor IF2	translation initiation factor IF-2 [chloroplast], putative	At1g17220	cluster B1
unknown protein	hypothetical protein	At1g33860	cluster B1
DNA-binding protein, putative	myb family transcription factor	At1g01060	cluster B1
arginine decarboxylase	arginine decarboxylase 1 (SPE1)	At2g16500	cluster B1
subtilisin proteinase like protein	subtilisin-like serine protease	At4g21650	cluster B1
unknown protein	cell wall protein precursor, putative	At2g20870	cluster B1
unknown protein	glycosyl hydrolase family 5/cellulase	At5g01930	cluster B1
lycopene epsilon cyclase	lycopene epsilon cyclase	At5g57030	cluster B1
putative protease SppA (SppA)	SppA protease IV (SppA)	At1g73990	cluster B1
putative protein	expressed protein	At3g61700	cluster B1
putative copper/zinc superoxide dismutase	copper/zinc superoxide dismutase (CSD2)	At2g28190	cluster B1
unknown protein	actin-related protein 4 (ARP4)	At1g18450	cluster B1
unknown protein	sporulation protein AA -related	At3g10420	cluster B1
AP2 domain containing protein RAP2.3	AP2 domain transcription factor RAP2.3	At3g16770	cluster B1
unknown protein	expressed protein	At2g47270	cluster B1
unknown protein	pectinesterase family	At1g14890	cluster B1
germin-like protein (GLP2a) copy2	germin-like protein (AtGER2)	At5g39190	cluster B1
putative Cu/Zn superoxide dismutase copper chaperone	copper/zinc superoxide dismutase copper chaperone, putative	At1g12520	cluster B1
putative protein	expressed protein	At1g70230	cluster B1
unknown protein	surp module (suppressor-of-white-APricot splicing regulator)	At5g23080	cluster B1
"acyl-(acyl carrier protein) thioesterase, putative"	acyl-(acyl carrier protein) thioesterase	At1g08510	cluster B1
"receptor-like kinase, putative"	leucine rich repeat protein kinase family	At3g23750	cluster B1
ethylene-insensitive3-like1 (EIL1)	ethylene-insensitive3-like1 (EIL1)	At2g27050	cluster B1
hsp 70-like protein	heat shock protein cpHsc70-1	At4g24280	cluster B1
unknown protein	late embryogenesis abundant (LEA) protein family	At4g02380	cluster B1
ribosomal protein S7	hypothetical prorein	At5g35130	cluster B1
psal	PSI I protein	At5g51790	cluster B1
putative protein	aldehyde dehydrogenase, putative (ALDH)	At1g54100	cluster B2
lipid-transfer protein-like	protease inhibitor/seed storage/lipid transfer protein(LTP)	At3g43720	cluster B2
cinnamoyl-CoA reductase - like protein	cinnamoyl-CoA reductase-related	At4g30470	cluster B2
GDSL-motif lipase/hydrolase-like protein	GDSL-motif lipase/hydrolase protein family	At5g45950	cluster B2
nucleoid DNA-binding - like protein	nucleoid DNA-binding - like protein	At3g54400	cluster B2
unknown protein	dehydrin (ERD14)	At1g76180	cluster B2
14-3-3 protein homolog RCI1	14-3-3 protein GF14 psi (grf3/RCI1)	At5g38480	cluster B2
protein phosphatase 2C ABI2 (PP2C)	protein phosphatase 2C, ABI2	At5g57050	cluster B2
abscisic acid insensitive protein (ABI1)	protein phosphatase ABI1	At4g26080	cluster B2
unknown protein	expressed protein	At4g25670	cluster B2
unknown protein	expressed protein	At1g19180	cluster B2
predicted protein of unknown function	TolB protein -related	At4g01870	cluster B2
putative mRNA capping enzyme	mRNA capping enzyme-related	At3g09100	cluster B2
ABC transporter like protein	ABC transporter family protein	At5g06530	cluster B2
integral membrane protein, putative	MATE efflux protein family	At3g21690	cluster B2
1-aminocyclopropane-1-carboxylate oxidase	abscisic acid responsive elements-binding factor(ABF3	At5g43450	cluster B2
unknown protein	expressed protein	At5g56980	cluster B2
cinnamyl-alcohol dehydrogenase ELI3-1	mannitol dehydrogenase (ELI3-1), putative	At4g37980	cluster B2
4-coumarate-CoA ligase -like protein	AMP-dependent synthetase and ligase family	At3g48990	cluster B2
unknown protein	VQ motif-containing protein family	At3g22160	cluster B2
ethylene response sensor (ERS)	ethylene response sensor (ERS)	At2g40940	cluster B2
ATP-dependent transmembrane transporter, putative	ABC transporter family protein	At1g51460	cluster B2
formate dehydrogenase (FDH)	formate dehydrogenase (FDH)	At5g14780	cluster B2
hypothetical protein	RNA binding protein 47 (RBP47), putative	At1g47500	cluster B2
Unknown protein	expressed protein	At5g27320	cluster B2
1-aminocyclopropane-1-carboxylate oxidase	1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase)	At1g05010	cluster B2
RSH3	RelA/SpoT protein, putative (RSH3)	At1g54130	cluster B2
putative protein	expressed protein	At3g59900	cluster B2

Table 1 continued:

Description (MIPS)	Description (TIGR)	Accession	Cluster
UDP-glucose:indole-3-acetate beta-D-glucosyltransferase(iaglu)	UDP-glucose:indole-3-acetate beta-D-glucosyltransferase (iaglu)	At4g15550	cluster B2
anthranilate N-benzoyltransferase - like protein	transferase family	At5g01210	cluster B2
putative ethylene receptor ERS2	ethylene receptor-related	At1g04310	cluster B2
unknown protein	transport protein Gsa12p -related	AAt3g62770	cluster B2
unknown protein	expressed protein	At5g18460	cluster B2
Nitrilase 4 (sp|P46011)	nitrilase 4 (NIT4)	At5g22300	cluster B2
unknown	expressed protein	At1g53890	cluster B2
ethylene responsive element binding factor 2 (ATERF2)	ethylene responsive element binding factor 2 (EREBP-2)	At5g47220	cluster B2
Unknown protein (K15C23.2)	expressed protein	At5g44580	cluster B2
unknown protein	brassinosteroid signalling positive regulator, putative	At1g19350	cluster B2
hypothetical protein	calcium-binding EF-hand protein family	At1g54450	cluster B2
putative protein	hydrolase family	At4g33540	cluster B2
unknown protein	expressed protein	At4g27900	cluster B2
60S ribosomal protein L30	60S ribosomal protein L24, putative	At2g44860	cluster B2
ribosomal protein L36-like	ribosomal protein family	At5g20180	cluster B2
ids4-like protein	SPX (SYG1/Pho81/XPR1) domain protein	At5g20150	cluster B2
putative fibrillin	plastid-lipid associated protein PAP/fibrillin, putative	At4g04020	cluster B2
abscisic acid responsive elements-binding factor(ABRE/ABF3)	AtbZip37Zip transcription factor AtbZip37	At4g34000	cluster B2
receptor kinase (CLV1)	CLAVATA1 receptor kinase (CLV1)	At1g75820	cluster B2
PSI 9KDa protein	At4g14160	psaC	cluster B2
putative auxin-induced protein AUX2-11	auxin-responsive protein IAA3 (Indoleacetic acid-induced protein 3)	At1g04240	cluster C
expansin S2 precursor like protein	expansin, putative (EXP11)	At1g20190	cluster C
putative protein	acireductone dioxygenase (ARD) family	At5g43850	cluster C
putative protein	Kelch repeats protein family	At5g03010	cluster C
zinc finger like protein	zinc finger (CCCH type) protein family	At5g44260	cluster C
putative cysteine proteinase	cysteine proteinase XCP2	At1g20850	cluster C
putative protein	expressed protein	At5g16730	cluster C
unknown protein	unknown protein	At3g15100	cluster D
sugar transporter like protein	mannitol transporter, putative	At4g36670	cluster D
putative trypsin inhibitor (At1g73260)	trypsin and protease inhibitor (Kunitz) family	At1g73260	cluster D
"peroxidase, prxr2"	peroxidase, putative	At4g37520	cluster D
peroxidase ATP8a	peroxidase, putative	At4g30170	cluster D
two-pore calcium channel (AtTPC1)	two-pore calcium channel (TPC1)	At4g03560	cluster D
phosphoenolpyruvate carboxykinase (ATP) -like protein	phosphoenolpyruvate carboxykinase [ATP], putative	At4g37870	cluster D
putative protein	polygalacturonase (pectinase) family	At1g70370	cluster D
putative protein kinase	protein kinase family	At3g56050	cluster D
nodulin-like protein	nodulin MtN21 family protein	At1g75500	cluster D
cytochrome c oxidoreductase like protein	nodulin MtN3 family protein	At4g15920	cluster D
unknown protein	expressed protein	At5g62130	cluster D
Fe(II) transport protein	iron-regulated transporter (IRT1)	At4g19690	cluster D
expansin At-EXP5	expansin, putative (EXP5)	At3g29030	cluster D
germin-like protein	germin-like protein (GLP4) (GLP5)	At1g09560	cluster D
nicotianamine synthase, putative	nicotianamine synthase	At5g04950	cluster D
lateral organ boundaries (LOB) domain protein 25 (LBD25)	unknown protein	At3g27650	cluster D
glycine-rich protein	unknown protein	At2g36120	cluster D
glycosyl hydrolase family 17	beta-1,3-glucanase class I precursor	At4g16260	cluster D
osmotin-like protein (OSM34)	osmotin precursor	At4g11650	cluster D
photoassimilate-responsive protein -related	photoassimilate-responsive protein PAR-1b -like protein	At3g54040	cluster D
auxin efflux carrier protein family	unknown protein	At2g17500	cluster D
integral membrane protein family	unknown protein	At4g27860	cluster D
DNA binding protein GT-1 -related	"putative DNA-binding protein, GT-1	At3g25990	cluster D
proline-rich protein family	putative protein	At4g38080	cluster D
meprin and TRAF homology (MATH) domain protein	unknown protein	At5g26260	cluster D
cytochrome p450 family	cytochrome P450-like protein	At5g48000	cluster D
peroxidase, putative	peroxidase ATP13a	At5g17820	cluster D
aminotransferase-related	hypothetical protein	At4g28410	cluster D
jacalin lectin family	putative lectin	At3g16390	cluster D
flavonol synthase, putative	1-aminocyclopropane-1-carboxylic acid oxidase-like protein	At5g63600	cluster D
protease inhibitor/seed storage/lipid transfer protein (LTP)	unknown protein	At2g48140	cluster D
myrosinase binding protein, putative	putative lectin	At3g16420	cluster D
jacalin lectin family	putative myrosinase-binding protein	At2g39310	cluster D
glycosyl hydrolase family	thioglucosidase 3D precursor	At3g09260	cluster D
glutamate decarboxylase 1 (GAD 1)	glutamate decarboxylase 1 (GAD 1)	At5g17330	cluster D
jacalin lectin family	putative jasmonate inducible protein	At3g16400	cluster D
FAD-linked oxidoreductase family	unknown protein	At2g46750	cluster D
meprin and TRAF homology (MATH) domain protein	unknown protein	At5g26280	cluster D
protein kinase family	receptor-protein kinase-like protein	At5g24010	cluster D
disease resistance response protein-related	disease resistance response/ dirigent - like protein	At3g55230	cluster D
glycosyl hydrolase family 19 (chitinase)	putative endochitinase	At2g43610	cluster D
short-chain dehydrogenase/reductase family protein	putative alcohol dehydrogenase	At2g47130	cluster D
uclacyanin II	phytocyanin, blue copper-binding protein II	At2g44790	cluster D
glyceraldehyde 3-phosphate dehydrogenase, putative	putative protein	At1g79530	cluster D
esterase/lipase/thioesterase family	phospholipase like protein	At2g39420	cluster D
inorganic phosphate transporter (PHT1)	phosphate transporter (gb|AAB17265.1)	At5g43350	cluster D

Table 1 continued:

Description (MIPS)	Description (TIGR)	Accession	Cluster
glycosyl hydrolase family 1	beta-glucosidase, putative	At1g66280	cluster D
peroxidase, putative	putative peroxidase	At3g21770	cluster D
patatin, putative	patatin-like protein	At4g37070	cluster D
peroxidase, putative	peroxidase ATP3a (emb|CAA67340.1)	At5g64100	cluster D
pathogen-inducible alpha-dioxygenase, putative	feebly like protein	At3g01420	cluster D
iron superoxide dismutase (FSD1)	superoxide dismutase (EC 1.15.1.1) (Fe)	At4g25100	cluster D
meprin and TRAF homology (MATH) domain protein	unknown protein	At5g26290	cluster D
expressed protein	unknown protein	At1g47480	cluster D
disease resistance protein (TIR class), putative	putative protein	At5g44920	cluster D
major latex protein (MLP)-related	unknown protein	At2g01520	cluster D
cinnamoyl-CoA reductase, putative	cinnamoyl CoA reductase like protein	At1g80820	cluster D
peroxidase, putative (ATP2a)	putative peroxidase ATP2a	At2g37130	cluster D
WRKY family transcription factor	WRKY transcription factor 11 (WRKY11)	At4g31550	cluster D
protease inhibitor, putative (Dr4)	putative protein	At1g73330	cluster D
expressed protein	hypothetical protein	At3g21710	cluster D
glycosyltransferase family	UTP-glucose glucosyltransferase	At5g66690	cluster D
disease resistance response protein-related	putative protein	At4g13580	cluster D
proline-rich protein family	periaxin - like protein	At5g09530	cluster D
phosphoenolpyruvate carboxylase, putative	phosphoenolpyruvate carboxylase (PPC)	At3g14940	cluster D
nitrate/chlorate transporter (NRT1.1 / CHL1)	nitrate/chlorate transporter CHL1	At1g12110	cluster D
pfkB type carbohydrate kinase protein family	putative fructokinase	At2g31390	cluster D
MuDR (mutator) transposase family	unknown protein	At5g50315	cluster D
catalase 3	unknown protein	At1g20620	cluster D
myrosinase-associated protein, putative	unknown protein	At1g54000	cluster D
laccase (diphenol oxidase), putative	putative laccase	At2g30210	cluster D
amino acid permease family	putative protein	At3g13620	cluster D
ABC transporter family protein	ABC transporter - like protein	At3g53480	cluster D
cytochrome P450, putative	cytochrome P450 monooxygenase like protein	At4g37410	cluster D
glycosyl hydrolase family 32	putative beta-fructosidase (At1g62660)	At1g62660	cluster D
2-oxoglutarate-dependent dioxygenase, putative	putative Iron/Ascorbate oxidoreductase family protein	At1g06640	cluster D
amino acid permease 2 (AAP2)	amino acid transport protein AAP2	At5g09220	cluster D
trypsin inhibitor, putative	putative trypsin inhibitor	At2g43510	cluster D
putative trypsin inhibitor	putative trypsin inhibitor	ATAC002335	cluster D
expressed protein	unknown protein	At4g22212	cluster D
peroxidase, putative	peroxidase (emb|CAA66960.1)	At5g42180	cluster D
cytochrome P450 90C1 / rotundifolia3 (rot3)	cytochrome P450 (ROTUNDIFOLIA3)	At4g36380	cluster D
1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase), putative	1-aminocyclopropane-1-carboxylate oxidase	At2g19590	cluster D
zinc transporter (ZIP3)	putative Fe(II) transporter	At2g32270	cluster D
myrosinase-associated protein, putative	unknown protein	At1g54010	cluster D
ferredoxin--NADP(+) reductase (adrenodoxin reductase), putative	ferrodoxin NADP oxidoreductase - like protein	At1g30510	cluster D
pyruvate decarboxylase, putative	pyruvate decarboxylase-1 (Pdc1)	At4g33070	cluster D
peroxidase	peroxidase like protein	At2g38380	cluster D
thioredoxin H-type 3 (TRX-H-3)	thioredoxin (clone GIF1) (pir||S58118)	At5g42980	cluster D
peroxidase, putative	putative peroxidase ATP12a	At1g05240	cluster D
early nodulin ENOD18 family	unknown protein	At3g03270	cluster D
pectin methylesterase, putative	unknown protein	At1g11580	cluster D
acyltransferase family	HSR201 like protein	At4g15390	cluster D
methionine sulfoxide reductase (SelR) domain protein	putative protein	At4g21850	cluster D
sugar transporter, putative	putative hexose transporter	At4g02050	cluster D
expressed protein	putative protein	At5g65480	cluster D
SWIB complex BAF60b domain-containing protein	Unknown protein (At2g14880;T26I20.4)	At2g14880	cluster D
aldose 1-epimerase family	possible apospory-associated like protein	At4g25900	cluster D
heat shock protein 101 (HSP101)	heat shock protein 101	At1g74310	cluster D

Table 2: Overview of ethylene-regulated genes from the microarray analysis according to the AQBC clustering

Description (MIPS)	Description (TIGR)	Accession	Cluster
putative peroxidase ATP2a	peroxidase, putative (ATP2a)	At2g37130	cluster 1
1-aminocyclopropane-1-carboxylic acid oxidase-like	flavonol synthase, putative	At5g63600	cluster 1
cytochrome P450 (ROTUNDIFOLIA3)	cytochrome P450 90C1 / rotundifolia3 (rot3)	At4g36380	cluster 1
peroxidase like protein	peroxidase	At2g38380	cluster 1
ABC transporter - like protein	ABC transporter family protein	At3g53480	cluster 1
Fe(II) transport protein	iron-regulated transporter (IRT1)	At4g19690	cluster 1
unknown protein	major latex protein (MLP)-related	At2g01520	cluster 1
putative myrosinase-binding protein	jacalin lectin family	At2g39310	cluster 1
unknown protein	FAD-linked oxidoreductase family	At2g46750	cluster 1
putative peroxidase	peroxidase, putative	At3g21770	cluster 1
phosphate transporter (gb|AAB17265.1)	inorganic phosphate transporter (PHT1)	At5g43350	cluster 1
feebly like protein	pathogen-inducible alpha-dioxygenase, putative	At3g01420	cluster 1
nitrate/chlorate transporter CHL1	nitrate/chlorate transporter (NRT1.1 / CHL1)	At1g12110	cluster 1
putative protein	glyceraldehyde 3-phosphate dehydrogenase	At1g79530	cluster 1
putative protein	disease resistance protein (TIR class), putative	At5g44920	cluster 1
beta-1,3-glucanase class I precursor	glycosyl hydrolase family 17	At4g16260	cluster 1
peroxidase ATP3a (emb|CAA67340.1)	peroxidase, putative	At5g64100	cluster 1
cytochrome P450 monooxygenase like protein	cytochrome P450, putative	At4g37410	cluster 1
thioredoxin (clone GIF1) (pir||S58118)	thioredoxin H-type 3 (TRX-H-3)	At5g42980	cluster 1
putative alcohol dehydrogenase	short-chain dehydrogenase/reductase family protein	At2g47130	cluster 1
putative protein kinase	protein kinase family	At3g56050	cluster 1
WRKY transcription factor 11 (WRKY11)	WRKY family transcription factor	At4g31550	cluster 1
putative Fe(II) transporter	zinc transporter (ZIP3)	At2g32270	cluster 1
unknown protein	FAD-linked oxidoreductase family	At2g46750	cluster 1
cytochrome c oxidoreductase like protein	nodulin MtN3 family protein	At4g15920	cluster 1
thioglucosidase 3D precursor	glycosyl hydrolase family	At3g09260	cluster 1
superoxide dismutase (EC 1.15.1.1) (Fe)	iron superoxide dismutase (FSD1)	At4g25100	cluster 1
unknown protein	catalase 3	At1g20620	cluster 1
putative trypsin inhibitor	putative trypsin inhibitor	ATAC002335	cluster 1
phosphoenolpyruvate carboxylase (PPC)	phosphoenolpyruvate carboxylase (PEPCase), putative	At3g14940	cluster 1
cinnamoyl CoA reductase like protein	cinnamoyl-CoA reductase, putative	At1g80820	cluster 1
photoassimilate-responsive protein PAR-1b -like protein	photoassimilate-responsive protein -related	At3g54040	cluster 1
putative protein	methionine sulfoxide reductase (SelR) domain protein	At4g21850	cluster 1
beta-glucosidase, putative	glycosyl hydrolase family 1	At1g66280	cluster 1
glutamate decarboxylase 1 (GAD 1) (sp|Q42521)	glutamate decarboxylase 1 (GAD 1)	At5g17330	cluster 1
unknown protein	expressed protein	At4g22212	cluster 1
putative protein	amino acid permease family	At3g13620	cluster 1
putative laccase	laccase (diphenol oxidase), putative	At2g30210	cluster 1
phytocyanin, blue copper-binding protein II	uclacyanin II	At2g44790	cluster 1
unknown protein	meprin and TRAF homology (MATH) domain protein	At5g26260	cluster 1
putative protein	disease resistance response protein-related	At4g13580	cluster 1
unknown protein	glycine-rich protein	At2g36120	cluster 1
putative jasmonate inducible protein	jacalin lectin family	At3g16400	cluster 1
putative trypsin inhibitor	trypsin inhibitor, putative	At2g43510	cluster 1
hypothetical protein	expressed protein	At3g21710	cluster 1
unknown protein	myrosinase-associated protein, putative	At1g54010	cluster 1
cytochrome P450-like protein	cytochrome p450 family	At5g48000	cluster 1
patatin-like protein	patatin, putative	At4g37070	cluster 1
putative lectin	jacalin lectin family	At3g16390	cluster 1
peroxidase (emb|CAA66960.1)	peroxidase, putative	At5g42180	cluster 1
unknown protein	meprin and TRAF homology (MATH) domain protein	At5g26290	cluster 1
unknown protein	early nodulin ENOD18 family	At3g03270	cluster 1
putative Iron/Ascorbate oxidoreductase family protein	2-oxoglutarate-dependent dioxygenase, putative	At1g06640	cluster 1
periaxin - like protein	proline-rich protein family	At5g09530	cluster 1
unknown protein	myrosinase-associated protein, putative	At1g54000	cluster 1
putative protein	proline-rich protein family	At4g38080	cluster 1
UTP-glucose glucosyltransferase	glycosyltransferase family	At5g66690	cluster 1
nitrate/chlorate transporter CHL1	nitrate/chlorate transporter (NRT1.1 / CHL1)	At1g12110	cluster 1
unknown protein	expressed protein	At1g47480	cluster 1
putative lectin	myrosinase binding protein, putative	At3g16420	cluster 1
putative hexose transporter	sugar transporter, putative	At4g02050	cluster 1
unknown protein	integral membrane protein family	At4g27860	cluster 1
amino acid transport protein AAP2	amino acid permease 2 (AAP2)	At5g09220	cluster 1
receptor-protein kinase-like protein	protein kinase family	At5g24010	cluster 1
unknown protein	meprin and TRAF homology (MATH) domain protein	At5g26260	cluster 1
unknown protein	meprin and TRAF homology (MATH) domain protein	At5g26280	cluster 1
putative protein	protease inhibitor, putative (Dr4)	At1g73330	cluster 1
germin-like protein	germin-like protein (GLP4) (GLP5)	At1g09560	cluster 1
"putative DNA-binding protein, GT-1"	DNA binding protein GT-1 -related	At3g25990	cluster 1
unknown protein	protease inhibitor/seed storage/lipid transfer protein (LTP)	At2g48140	cluster 1
peroxidase ATP8a	peroxidase, putative	At4g30170	cluster 1
putative fructokinase	pfkB type carbohydrate kinase protein family	At2g31390	cluster 1
ferrodoxin NADP oxidoreductase - like protein	ferredoxin--NADP(+) reductase, putative	At1g30510	cluster 1
putative peroxidase ATP12a	peroxidase, putative	At1g05240	cluster 1
nicotianamine synthase (dbj|BAA74589.1)	nicotianamine synthase, putative	At5g04950	cluster 1
unknown protein	auxin efflux carrier protein family	At2g17500	cluster 1
HSR201 like protein	acyltransferase family	At4g15390	cluster 1
peroxidase ATP13a	peroxidase, putative	At5g17820	cluster 1
phosphoenolpyruvate carboxykinase (ATP) -like protein	phosphoenolpyruvate carboxykinase [ATP], putative	At4g37870	cluster 1

Table 2 continued:

Description (MIPS)	Description (TIGR)	Accession	Cluster
disease resistance response/ dirigent - like protein	disease resistance response protein-related	At3g55230	cluster 1
unknown protein	pectin methylesterase, putative	At1g11580	cluster 1
unknown protein	expressed protein	At5g62130	cluster 1
putative endochitinase	glycosyl hydrolase family 19 (chitinase)	At2g43610	cluster 1
phospholipase like protein	esterase/lipase/thioesterase family	At2g39420	cluster 1
putative beta-fructosidase (At1g62660)	glycosyl hydrolase family 32	At1g62660	cluster 1
putative fructokinase	pfkB type carbohydrate kinase protein family	At2g31390	cluster 1
putative fructokinase	pfkB type carbohydrate kinase protein family	At2g31390	cluster 1
unknown protein	MuDR (mutator) transposase family	At5g50315	cluster 1
osmotin precursor	osmotin-like protein (OSM34)	At4g11650	cluster 1
putative trypsin inhibitor	trypsin inhibitor, putative	At2g43510	cluster 1
unknown protein	lateral organ boundaries (LOB) domain protein 25 (LBD25)	At3g27650	cluster 1
putative protein	expressed protein	At1g70230	cluster 2
Nitrilase 4 (sp|P46011)	nitrilase 4 (NIT4)	At5g22300	cluster 2
predicted protein of unknown function	TolB protein -related	At4g01870	cluster 2
putative mRNA capping enzyme, RNA guanylyltransferase	mRNA capping enzyme-related	At3g09100	cluster 2
putative ethylene receptor ERS2	ethylene receptor-related	At1g04310	cluster 2
putative protein	expressed protein	At3g61700	cluster 2
unknown protein	expressed protein	At4g27900	cluster 2
putative protein	hydrolase family	At4g33540	cluster 2
putative protein	expressed protein	At3g59900	cluster 2
unknown protein	brassinosteroid signalling positive regulator, putative	At1g19350	cluster 2
60S ribosomal protein L30	60S ribosomal protein L24, putative	At2g44860	cluster 2
Unknown protein	expressed protein	At5g27320	cluster 2
1-aminocyclopropane-1-carboxylate oxidase	abscisic acid responsive elements-binding factor(ABF3	At5g43450	cluster 2
putative copper/zinc superoxide dismutase	copper/zinc superoxide dismutase (CSD2)	At2g28190	cluster 2
cinnamyl-alcohol dehydrogenase ELI3-1	mannitol dehydrogenase (ELI3-1), putative	At4g37980	cluster 2
ethylene responsive element binding factor 2 (ATERF2)	ethylene responsive element binding factor 2 (EREBP-2)	At5g47220	cluster 2
hypothetical protein	RNA binding protein 47 (RBP47), putative	At1g47500	cluster 2
unknown protein	expressed protein	At1g19180	cluster 2
unknown protein	brassinosteroid signalling positive regulator, putative	At1g19350	cluster 2
4-coumarate-CoA ligase -like protein	AMP-dependent synthetase and ligase family	At3g48990	cluster 2
unknown protein	expressed protein	At5g18460	cluster 2
UDP-glucose:indole-3-acetate beta-D-glucosyltransferase (iaglu)	UDP-glucose:indole-3-acetate beta-D-glucosyltransferase	At4g15550	cluster 2
ethylene response sensor (ERS)	ethylene response sensor (ERS)	At2g40940	cluster 2
abscisic acid responsive elements-binding factor (ABRE/ABF3)	bZip transcription factor AtbZip37	At4g34000	cluster 2
anthranilate N-benzoyltransferase - like protein	transferase family	At5g01210	cluster 2
"ATP-dependent transmembrane transporter, putative"	ABC transporter family protein	At1g51460	cluster 2
Unknown protein	expressed protein	At5g27320	cluster 2
hypothetical protein	calcium-binding EF-hand protein family	At1g54450	cluster 2
unknown protein	VQ motif-containing protein family	At3g22160	cluster 2
ids4-like protein	SPX (SYG1/Pho81/XPR1) domain protein	At5g20150	cluster 2
1-aminocyclopropane-1-carboxylate oxidase	1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase)	At1g05010	cluster 2
unknown	expressed protein	At1g53890	cluster 2
RSH3	RelA/SpoT protein, putative (RSH3)	At1g54130	cluster 2
ribosomal protein L36-like	ribosomal protein family	At5g20180	cluster 2
Unknown protein (K15C23.2)	expressed protein	At5g44580	cluster 2
formate dehydrogenase (FDH)	formate dehydrogenase (FDH)	At5g14780	cluster 2
unknown protein	transport protein Gsa12p -related	At3g62770	cluster 2
ethylene response sensor (ERS)	ethylene response sensor (ERS)	At2g40940	cluster 2
acyl-(acyl carrier protein) thioesterase, putative	acyl-(acyl carrier protein) thioesterase	At1g08510	cluster 3
putative Cu/Zn superoxide dismutase copper chaperone	copper/zinc superoxide dismutase copper chaperone, put.	At1g12520	cluster 3
unknown protein	actin-related protein 4 (ARP4)	At1g18450	cluster 3
ethylene-insensitive3-like1 (EIL1)	ethylene-insensitive3-like1 (EIL1)	At2g27050	cluster 3
AP2 domain containing protein RAP2.3	AP2 domain transcription factor RAP2.3	At3g16770	cluster 3
subtilisin proteinase like protein	subtilisin-like serine protease	At4g21650	cluster 3
unknown protein	cell wall protein precursor, putative	At2g20870	cluster 3
putative translation initiation factor IF2	translation initiation factor IF-2 [chloroplast], putative	At1g17220	cluster 3
plasma membrane proton ATPase-like	ATPase, plasma membrane-type (proton pump), putative	At5g62670	cluster 3
elongation factor G, putative	elongation factor Tu family protein	At1g62750	cluster 3
DNA-binding protein, putative	myb family transcription factor	At1g01060	cluster 3
GTP-binding protein typA (tyrosine phosphorylated protein A)	GTP-binding protein typA	At5g13650	cluster 3
hsp 70-like protein	heat shock protein cpHsc70-1	At4g24280	cluster 3
xyloglucan endo-1,4-beta-D-glucanase-like protein	xyloglucan endotransglycosylase, putative	At4g30280	cluster 3
putative protein	WD-40 repeat protein family	At1g15750	cluster 3
unknown protein	sporulation protein AA -related	At3g10420	cluster 3
putative SF16 protein {Helianthus annuus}	calmodulin-binding protein family	At2g43680	cluster 3
unknown protein	glycosyl hydrolase family 5/cellulase	At5g01930	cluster 3
germin-like protein (GLP2a) copy2	germin-like protein (AtGER2)	At5g39190	cluster 3
lycopene epsilon cyclase	lycopene epsilon cyclase	At5g57030	cluster 3
arginine decarboxylase	arginine decarboxylase 1 (SPE1)	At2g16500	cluster 3
putative protein	expressed protein	At1g80865	cluster 3
unknown protein	elongation factor Ts family	At4g29060	cluster 3
receptor-like kinase, putative	leucine rich repeat protein kinase family	At3g23750	cluster 3
unknown protein	hypothetical protein	At1g33860	cluster 3
putative receptor-like protein kinase, ERECTA	leucine rich repeat protein kinase family (ERECTA)	At2g26330	cluster 3
GTP-binding protein typA (tyrosine phosphorylated protein A)	GTP-binding protein typA	At5g13650	cluster 3
arabinogalactan-protein AGP1 (gb|AAC77823.1)	arabinogalactan-protein (AGP1)	At5g64310	cluster 3
integral membrane protein, putative	MATE efflux protein family	At3g21690	cluster 4
receptor kinase (CLV1)	CLAVATA1 receptor kinase (CLV1	At1g75820	cluster 4

Table 2 continued:

Description (MIPS)	Description (TIGR)	Accession	Cluster
abscisic acid insensitive protein (ABI1)	protein phosphatase ABI1	At4g26080	cluster 4
unknown protein	expressed protein	At4g25670	cluster 4
protein phosphatase 2C ABI2 (PP2C) (sp|O04719)	protein phosphatase 2C, ABI2	At5g57050	cluster 4
14-3-3 protein homolog RCI1 (pir||S47969)	14-3-3 protein GF14 psi (grf3/RCI1)	At5g38480	cluster 4
abscisic acid insensitive protein (ABI1)	protein phosphatase ABI1	At4g26080	cluster 4
unknown protein	dehydrin (ERD14)	At1g76180	cluster 4
S-adenosylmethionine decarboxylase (adoMetDC2)	adenosylmethionine decarboxylase family	At5g15950	cluster 4
unknown protein	expressed protein	At5g56980	cluster 4
Unknown protein (At2g14880;T26I20.4)	SWIB complex BAF60b domain-containing protein	At2g14880	cluster 5
putative protein	expressed protein	At5g65480	cluster 5
expansin At-EXP5	expansin, putative (EXP5)	At3g29030	cluster 5
expansin At-EXP5	expansin, putative (EXP5)	At3g29030	cluster 5
nodulin-like protein	nodulin MtN21 family protein	At1g75500	cluster 5
two-pore calcium channel (AtTPC1)	two-pore calcium channel (TPC1)	At4g03560	cluster 5
putative protein	polygalacturonase (pectinase) family	At1g70370	cluster 5
peroxidase, prxr2	peroxidase, putative	At4g37520	cluster 5
putative auxin-induced protein AUX2-11	auxin-responsive protein IAA3	At1g04240	cluster 5
ABC transporter like protein	ABC transporter family protein	At5g06530	cluster 6
lipid-transfer protein-like, predicted GPI-anchored protein	protease inhibitor/seed storage/lipid transfer protein (LTP)	At3g43720	cluster 6
putative fibrillin	plastid-lipid associated protein PAP/fibrillin, putative	At4g04020	cluster 6
GDSL-motif lipase/hydrolase-like protein	GDSL-motif lipase/hydrolase protein family	At5g45950	cluster 6
cinnamoyl-CoA reductase - like protein	cinnamoyl-CoA reductase-related	At4g30470	cluster 6

4.10 REFERENCES

Alonso, J.M., Stepanova, A.N., Solano, R., Wisman, E., Ferrari, S., Ausubel, F.M., and Ecker, J.R. (2003). Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in Arabidopsis. Proc Natl Acad Sci U S A **100,** 2992-2997.

Asai, T., Stone, J.M., Heard, J.E., Kovtun, Y., Yorgey, P., Sheen, J., and Ausubel, F.M. (2000). Fumonisin B1-induced cell death in arabidopsis protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signalling pathways. Plant Cell **12,** 1823-1836.

Beaudoin, N., Serizet, C., Gosti, F., and Giraudat, J. (2000). Interactions between abscisic acid and ethylene signalling cascades. Plant Cell **12,** 1103-1115.

Bent, A.F., Innes, R.W., Ecker, J.R., and Staskawicz, B.J. (1992). Disease development in ethylene-insensitive Arabidopsis thaliana infected with virulent and avirulent Pseudomonas and Xanthomonas pathogens. Mol Plant Microbe Interact **5,** 372-378.

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. Plant J **29,** 23-32.

Bleecker, A.B., Estelle, M.A., Somerville, C., and Kende, H. (1988). Insensitivity to ethylene conferred by a dominant mutation in Arabidopsis thaliana seedlings. Science 241, 1086-1089.Brehelin, C., Mouaheb, N., Verdoucq, L., Lancelin, J.M., and Meyer, Y. (2000). Characterization of determinants for the specificity of Arabidopsis thioredoxins h in yeast complementation. J Biol Chem **275,** 31641-31647.

Chang, C. (2003). Ethylene signalling: the MAPK module has finally landed. Trends Plant Sci **8,** 365-368.

Cheong, Y.H., Chang, H.S., Gupta, R., Wang, X., Zhu, T., and Luan, S. (2002). Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. Plant Physiol **129,** 661-677.

Chisolm, S.T., Mahajan, S.K., Whitman, S.A., Yamamoto, M.L., and Carrington, J.C. (2000). Cloning of the Arabidopsis RTM1 gene, which controls restriction of long-distance movement of tobacco etch virus. Proc. Natl. Acad. Sci. U S A **97,** 489-494.

Choi, H., Hong, J., Ha, J., Kang, J., and Kim, S.Y. (2000). ABFs, a family of ABA-responsive element binding factors. J Biol Chem **275,** 1723-1730.

Claus, H. (2003). Laccases and their occurrence in prokaryotes. Arch Microbiol **179,** 145-150.

Cosgrove, D.J., Li, L.C., Cho, H.T., Hoffmann-Benning, S., Moore, R.C., and Blecker, D. (2002). The growing world of expansins. Plant Cell Physiol **43,** 1436-1444.

Czernic, P., Huang, H.C., and Marco, Y. (1996). Characterization of hsr201 and hsr515, two tobacco genes preferentially expressed during the hypersensitive reaction provoked by phytopathogenic bacteria. Plant Mol Biol **31,** 255-265.

Desikan, R., Griffiths, R., Hancock, J., and Neill, S. (2002). A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in Arabidopsis thaliana. Proc Natl Acad Sci U S A **99,** 16314-16318.

Dong, J., Chen, C., and Chen, Z. (2003). Expression profiles of the Arabidopsis WRKY gene superfamily during plant defence response. Plant Mol Biol **51,** 21-37.

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A **95,** 14863-14868.

Franceschetti, M., Hanfrey, C., Scaramagli, S., Torrigiani, P., Bagni, N., Burtin, D., and Michael, A.J. (2001). Characterization of monocot and dicot plant S-adenosyl-l-methionine decarboxylase gene families including identification in the mRNA of a highly conserved pair of upstream overlapping open reading frames. Biochem J **353,** 403-409.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H., and Ohme-Takagi, M. (2000). Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. Plant Cell **12,** 393-404.

Ghassemian, M., Nambara, E., Cutler, S., Kawaide, H., Kamiya, Y., and McCourt, P. (2000). Regulation of abscisic acid signalling by the ethylene response pathway in Arabidopsis. Plant Cell **12,** 1117-1126.

Gosti, F., Beaudoin, N., Serizet, C., Webb, A.A., Vartanian, N., and Giraudat, J. (1999). ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signalling. Plant Cell **11,** 1897-1910.

Greenberg, J.T., Silverman, F.P., and Liang, H. (2000). Uncoupling salicylic acid-dependent cell death and defence-related responses from disease resistance in the Arabidopsis mutant acd5. Genetics **156,** 341-350.

Guzman, P., and Ecker, J.R. (1990). Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell **2,** 513-523.

Hua, J., Sakai, H., Nourizadeh, S., Chen, Q.G., Bleecker, A.B., Ecker, J.R., and Meyerowitz, E.M. (1998). EIN4 and ERS2 are members of the putative ethylene receptor gene family in Arabidopsis. Plant Cell **10,** 1321-1332.

Jackson, R.G., Lim, E.K., Li, Y., Kowalczyk, M., Sandberg, G., Hoggett, J., Ashford, D.A., and Bowles, D.J. (2001). Identification and biochemical characterization of an Arabidopsis indole-3-acetic acid glucosyltransferase. J Biol Chem **276,** 4350-4356.

Johnson, P.R., and Ecker, J.R. (1998). The ethylene gas signal transduction pathway: a molecular perspective. Annu Rev Genet **32,** 227-254.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R. (1993). CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases. Cell **72,** 427-441.

Kiedrowski, S., Kawalleck, P., Hahlbrock, K., Somssich, I.E., and Dangl, J.L. (1992). Rapid activation of a novel plant defence gene is strictly dependent on the Arabidopsis RPM1 disease resistance locus. Embo J **11,** 4677-4684.

Lasserre, E., Bouquin, T., Hernandez, J.A., Bull, J., Pech, J.C., and Balague, C. (1996). Structure and expression of three genes encoding ACC oxidase homologs from melon (Cucumis melo L.). Mol Gen Genet **251,** 81-90.

Le Gourrierec, J., Li, Y.F., and Zhou, D.X. (1999). Transcriptional activation by Arabidopsis GT-1 may be through interaction with TFIIA-TBP-TATA complex. Plant J **18,** 663-668.

Lee, D., Ellard, M., Wanner, L.A., Davis, K.R., and Douglas, C.J. (1995). The Arabidopsis thaliana 4-coumarate:CoA ligase (4CL) gene: stress and developmentally regulated expression and nucleotide sequence of its cDNA. Plant Mol Biol **28,** 871-884.

Lellis, A.D., Kasschau, K.D., Whitham, S.A., and Carrington, J.C. (2002). Loss-of-susceptibility mutants of Arabidopsis thaliana reveal an essential role for eIF(iso)4E during potyvirus infection. Curr Biol **12,** 1046-1051.

Leon, P., and Sheen, J. (2003). Sugar and hormone connections. Trends Plant Sci **8,** 110-116.

Leung, J., Merlot, S., and Giraudat, J. (1997). The Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. Plant Cell **9,** 759-771.

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K.A., Dangl, J.L., and Dietrich, R.A. (2000). The transcriptome of Arabidopsis thaliana during systemic acquired resistance. Nat Genet **26,** 403-410.

Martinoia, E., Klein, M., Geisler, M., Bovet, L., Forestier, C., Kolukisaoglu, U., Muller-Rober, B., and Schulz, B. (2002). Multifunctionality of plant ABC transporters—more than just detoxifiers. Planta **214,** 345-355.

Moeder, W., Barry, C.S., Tauriainen, A.A., Betz, C., Tuomainen, J., Utriainen, M., Grierson, D., Sandermann, H., Langebartels, C., and Kangasjarvi, J. (2002). Ethylene synthesis regulated by biphasic induction of 1-aminocyclopropane-1-carboxylic acid synthase and 1-aminocyclopropane-1-carboxylic acid oxidase genes is required for hydrogen peroxide accumulation and cell death in ozone-exposed tomato. Plant Physiol **130,** 1918-1926.

Nakao, L.S., Iwai, L.K., Kalil, J., and Augusto, O. (2003). Radical production from free and peptide-bound methionine sulfoxide oxidation by peroxynitrite and hydrogen peroxide/iron(II). FEBS Lett **547,** 87-91.

Nersissian, A.M., Immoos, C., Hill, M.G., Hart, P.J., Williams, G., Herrmann, R.G., and Valentine, J.S. (1998). Uclacyanins, stellacyanins, and plantacyanins are distinct subfamilies of phytocyanins: plant-specific mononuclear blue copper proteins. Protein Sci **7,** 1915-1929.

Nylander, M., Svensson, J., Palva, E.T., and Welin, B.V. (2001). Stress-induced accumulation and tissue-specific localization of dehydrins in Arabidopsis thaliana. Plant Mol Biol **45,** 263-279.

Okamuro, J.K., Caster, B., Villarroel, R., Van Montagu, M., and Jofuku, K.D. (1997). The AP2 domain of APETALA2 defines a large new family of DNA binding proteins in Arabidopsis. Proc Natl Acad Sci U S A **94,** 7076-7081.

Overmyer, K., Tuominen, H., Kettunen, R., Betz, C., Langebartels, C., Sandermann, H., Jr., and Kangasjarvi, J. (2000). Ozone-sensitive arabidopsis rcd1 mutant reveals opposite roles for ethylene and jasmonate signalling pathways in regulating superoxide-dependent cell death. Plant Cell **12,** 1849-1862.

Penarrubia, L., Aguilar, M., Margossian, L., and Fischer, R.L. (1992). An Antisense Gene Stimulates Ethylene Hormone Production during Tomato Fruit Ripening. Plant Cell **4,** 681-687.

Penninckx, I.A., Eggermont, K., Terras, F.R., Thomma, B.P., De Samblanx, G.W., Buchala, A., Metraux, J.P., Manners, J.M., and Broekaert, W.F. (1996). Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. Plant Cell **8,** 2309-2323.

Piotrowski, M., Schonfelder, S., and Weiler, E.W. (2001). The Arabidopsis thaliana isogene NIT4 and its orthologs in tobacco encode beta-cyano-L-alanine hydratase/nitrilase. J Biol Chem **276,** 2616-2621.

Queitsch, C., Hong, S.W., Vierling, E., and Lindquist, S. (2000). Heat shock protein 101 plays a crucial role in thermotolerance in Arabidopsis. Plant Cell **12,** 479-492.

Roman, G., Lubarsky, B., Kieber, J.J., Rothenberg, M., and Ecker, J.R. (1995). Genetic analysis of ethylene signal transduction in Arabidopsis thaliana: five novel mutant loci integrated into a stress response pathway. Genetics **139,** 1393-1409.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., and Manners, J.M. (2000). Coordinated plant defence responses in Arabidopsis revealed by microarray analysis. Proc Natl Acad Sci U S A **97,** 11655-11660.

Schultz, C.J., Rumsewicz, M.P., Johnson, K.L., Jones, B.J., Gaspar, Y.M., and Bacic, A. (2002). Using genomic resources to guide research directions. The arabinogalactan protein gene family as a test case. Plant Physiol **129,** 1448-1463.

Smalle, J., Haegman, M., Kurepa, J., Van Montagu, M., and Van Der Straeten, D. (1997). Ethylene can stimulate Arabidopsis hypocotyl elongation in the light. Proc Natl Acad Sci U S A **94,** 2756-2761.

Sung, D.Y., Vierling, E., and Guy, C.L. (2001). Comprehensive expression profile analysis of the Arabidopsis Hsp70 gene family. Plant Physiol **126,** 789-800.

Taipalensuu, J., Falk, A., and Rask, L. (1996). A wound- and methyl jasmonate-inducible transcript coding for a myrosinase-associated protein with similarities to an early nodulin. Plant Physiol **110,** 483-491.

Thomma, B.P., Eggermont, K., Tierens, K.F., and Broekaert, W.F. (1999). Requirement of functional ethylene-insensitive 2 gene for efficient resistance of Arabidopsis to infection by Botrytis cinerea. Plant Physiol **121,** 1093-1102.

Tian, Q., Uhlir, N.J., and Reed, J.W. (2002). Arabidopsis SHY2/IAA3 inhibits auxin-regulated gene expression. Plant Cell **14,** 301-319.

Urwin, P.E., McPherson, M.J., and Atkinson, H.J. (1998). Enhanced transgenic plant resistance to nematodes by dual proteinase inhibitor constructs. Planta **204,** 472-479.

Vahala, J., Ruonala, R., Keinanen, M., Tuominen, H., and Kangasjarvi, J. (2003). Ethylene insensitivity modulates ozone-induced cell death in birch. Plant Physiol **132,** 185-195.

van den Brule, S., and Smart, C.C. (2002). The plant PDR family of ABC transporters. Planta **216,** 95-106.

van der Biezen, E.A., Sun, J., Coleman, M.J., Bibb, M.J., and Jones, J.D. (2000). Arabidopsis RelA/SpoT homologs implicate (p)ppGpp in plant signalling. Proc Natl Acad Sci U S A **97,** 3747-3752.

Vercauteren, I., Van Der Schueren, E., Van Montagu, M., and Gheysen, G. (2001). Arabidopsis thaliana genes expressed in the early compatible interaction with root-knot nematodes. Mol Plant Microbe Interact **14,** 288-299.

Verdoucq, L., Vignols, F., Jacquot, J.P., Chartier, Y., and Meyer, Y. (1999). In vivo characterization of a thioredoxin h target protein defines a new peroxiredoxin family. J Biol Chem **274,** 19714-19722.

Vogel, J.P., Schuerman, P., Woeste, K., Brandstatter, I., and Kieber, J.J. (1998a). Isolation and characterization of Arabidopsis mutants defective in the induction of ethylene biosynthesis by cytokinin. Genetics **149,** 417-427.

Waki, K., Shibuya, K., Yoshioka, T., Hashiba, T., and Satoh, S. (2001). Cloning of a cDNA encoding EIN3-like protein (DC-EIL1) and decrease in its mRNA level during senescence in carnation flower tissues. J Exp Bot **52,** 377-379.

Welinder, K.G., Justesen, A.F., Kjaersgard, I.V., Jensen, R.B., Rasmussen, S.K., Jespersen, H.M., and Duroux, L. (2002). Structural diversity and transcription of class III peroxidases from Arabidopsis thaliana. Eur J Biochem **269,** 6063-6081.

Wolfinger, R.D., Gibson, G., Wolfinger, E.D., Bennett, L., Hamadeh, H., Bushel, P., Afshari, C., and Paules, R.S. (2001). Assessing gene significance from cDNA microarray expression data via mixed models. J Comput Biol **8,** 625-637.

Yang, Y.H., Dutoit, S., Luu, p., Lin, D.M., Peng, V., Ngai, J., and Speed, T.P. (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systemic variation. Nucleic Acids Res **30:e15**.

Yang, Q., Reinhard, K., Schiltz, E., and Matern, U. (1997). Characterization and heterologous expression of hydroxycinnamoyl/benzoyl-CoA:anthranilate N-hydroxycinnamoyl/benzoyltransferase from elicited cell cultures of carnation, Dianthus caryophyllus L. Plant Mol Biol **35,** 777-789.

Zegzouti, H., Jones, B., Frasse, P., Marty, C., Maitre, B., Latch, A., Pech, J.C., and Bouzayen, M. (1999). Ethylene-regulated gene expression in tomato fruit: characterization of novel ethylene-responsive and ripening-related genes isolated by differential display. Plant J **18,** 589-600.

Zhou, L., Jang, J.C., Jones, T.L., and Sheen, J. (1998). Glucose and ethylene signal transduction crosstalk revealed by an Arabidopsis glucose-insensitive mutant. Proc Natl Acad Sci U S A **95,** 10294-10299.

PART II

Identification and analysis of *Arabidopsis* mutants with enhanced ethylene responses.

Chapter 5

The *Arabidopsis* mutant *eer2* has enhanced ethylene responses in the light

Chapter 5: The Arabidopsis mutant *eer2* has enhanced ethylene responses in the light

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5.1 SUMMARY

By screening for ethylene response mutants in *Arabidopsis*, we isolated a novel recessive mutant, *eer2,* which displays enhanced ethylene responses. Light-grown *eer2* seedlings showed an elongated hypocotyl on al low nutrient medium (LNM) to lower levels of ACC, the precursor of ethylene, compared to the wild type, indicating that *eer2* responds more sensitively to ethylene. Roots of *eer2* were somewhat less sensitive than the wild type to low concentrations of ACC. Treatment with MCP (methylcyclopropene), a competitive inhibitor of ethylene signalling, suppressed this hypersensitive response, demonstrating that the *eer2*-response is due to an ethylene-effect. The ethylene levels in this mutant were not increased. Thus, ethylene overproduction is not the primary cause of the *eer2*-phenotype. Besides its enhanced ethylene-response on the hypocotyls, *eer2* is also affected in its senescence pattern and its phenotype depends on the nutritional status of the growth-medium. Different functions for EER2 can be hypothesized.

5.2 INTRODUCTION

The gaseous hormone ethylene influences many aspects in growth and development of plants, including germination, cell elongation, flower and leaf senescence, fruit ripening, sex determination, and defence responses (Yang and Hoffman, 1984). Initiation of these processes involves regulation both of ethylene biosynthesis and perception. Many components of ethylene signalling have been identified, mainly through the use of the triple response (Guzman and Ecker, 1990). Indeed, dark-grown seedlings of *Arabidopsis* undergo conspicuous morphological changes in the presence of ethylene, consisting of a radial swelling of the hypocotyl, an exaggeration in the curvature of the apical hook, and an inhibition of cell elongation in the hypocotyl and root. This morphological ethylene-responsive changes have been termed the triple response. Three classes of ethylene response mutants were identified: insensitive mutants, constitutive response mutants, and those that are affected in an organ-specific manner (Bleecker et al., 1988; Kieber et al., 1993; Van Der Straeten et al., 1993; Roman et al., 1995). Cloning and characterization of genes in the ethylene signalling pathway provided insight in the molecular mechanisms

responsible for a plant's response to the gaseous hormone. The ethylene signal transduction chain in *Arabidopsis* as presently conceived, consists of five partially functionally redundant receptors, which negatively regulate ethylene signalling (Bleecker et al., 1988; Hua et al., 1995; Hua and Meyerowitz, 1998; Hua et al., 1998; Sakai et al., 1998). These receptors have significant similarity to His protein kinase receptors of two-component regulatory systems (Bleecker, 1999). Ethylene binding requires a copper cofactor as part of the functional receptor (Rodriguez et al., 1999), and a copper transporter protein, RAN1, is thought to provide copper ions to the receptors (Hirayama et al., 1999; Woeste and Kieber, 2000). Placed downstream of the ethylene receptors is CTR1, a Raf-like mitogen-activated protein kinase kinase kinase (MAPKKK) (Kieber et al., 1993). Direct evidence that a MAPK cascade is part of the ethylene signal transduction pathway in plants has been provided recently (Ouaked et al., 2003). *EIN2* acts downstream of *CTR1*; its aminoterminal half encodes an integral membrane protein that exhibits significant similarity to the Nramp family of cation transporters (Alonso et al., 1999). In response to ethylene, plants modulate the expression of specific genes at the transcriptional and post-transcriptional level (Lincoln and Fischer, 1988; Zegzouti et al., 1999; Koyama et al., 2001). Responses downstream of EIN2 are modulated by a two-step cascade of transcriptional regulators involving two families of transcription factors, the EIN3 (ethylene insensitive)/EILs (EIN3-like) proteins and the ERFs (ethylene response element binding factor) (Chao et al., 1997). *EIN3* is a nuclear-localized DNA-binding protein belonging to a small multigene family in *Arabidopsis.* Two EIN3-like proteins, *EIL1* and *EIL2*, could complement a loss-of-function mutation in EIN3, indicating that they are also involved in ethylene signal transduction. The immediate target of EIN3 is ERF1 which contains a primary ethylene response element (PERE) in its promotor (Solano et al., 1998). *ERF1* belongs to a large family of plant-specific transcription factors referred to as ERFs, which bind to the GCC box and activate the expression of secondary ethylene-response genes.

The standard triple response screen is likely saturated for the identification of viable loss-of-function mutants that affect ethylene responses. However, refinements of the screen continue to yield results. One refinement consisted in the screening for mutations that display an enhanced ethylene response at a low ethylene concentration. Using this screen, the enhanced-ethylene-response (*eer1*) mutant was isolated (Larsen and Chang, 2001). Molecular cloning of the *eer1* mutation revealed that it is a new allele of *RCN1*, an A regulatory subunit of PP2A (Larsen and Cancel, 2003). Furthermore, very recently, five components of the ethylene-response pathway were identified by screening at a low

concentration of the ethylene precursor (*wei1-wei5)* (weak ethylene-insensitive mutants) (Alonso et al., 2003). Novel screens might help to identify new ethylene-related loci apart from those implicated at the etiolated seedling stage. Two novel methods are based on the ethylene response in the light of nutrient-deficient seedlings at two stages of development. The first derives from the fact that ethylene induces hypocotyl elongation in the light (Smalle et al., 1997). The second is established of the finding that ethylene stimulates leaf emergence (Van Der Straeten, 1999). Using these novel assays, additional ethylene-related loci were discovered (Van Der Straeten et al., 1999; Vandenbussche et al., 2003).
Here, we describe a novel ethylene-related mutant, *eer2*, a second member of the class of enhanced ethylene response mutants, implying that the mutant displays a measurable ethylene response upon lower levels of ethylene than the wild type. Hypersensitive mutants have been also described for the ABA- and cytokinin-pathways (Cutler et al., 1996; Kubo and Kakimoto, 2000). A collection of mutants, named *era* (enhanced response to ABA), show enhanced responses to exogenous ABA. The *ERA* gene encodes the β-subunit of a protein farnesyl transferase (Cutler et al., 1996). The proteines encoded by the cytokinin-hypersensitive genes in *Arabidopsis*, *CKH1* and *CKH2*, are proposed to negatively regulate the cytokinin-signalling pathway for cell division and chloroplast development (Kubo and Kakimoto, 2000). Besides its hypersensitive response in ethylene-induced hypocotyl elongation, *eer2* also displays enhanced response at the molecular level and at later stages of development. Furthermore, *eer2* is affected in its senescence pattern and its phenotype depends on the nutritional status of the growth medium. Possible roles for EER2 are discussed.

5.3 RESULTS

5.3.1 Isolation and phenotypic characterization of *eer2*

By screening FN (fast neutrons)-mutagenized *Arabidopsis* for ethylene-signalling mutants on Low Nutrient Medium (LNM) in the presence of the ethylene precursor ACC in the light, we isolated a novel mutant, *eer2* (enhanced ethylene response), formerly named *fse1* for fast senescence ethylene-regulated mutant (De Paepe et al., 1999). Previous research indicated that the hypocotyl length of wild type Col-0 seedlings grown on LNM in the light, reaches up to twice the size of the untreated control in the presence of 50μM 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene (Smalle et al., 1997). For *eer2* a hypocotyl elongation of almost 100% was already visible in the presence

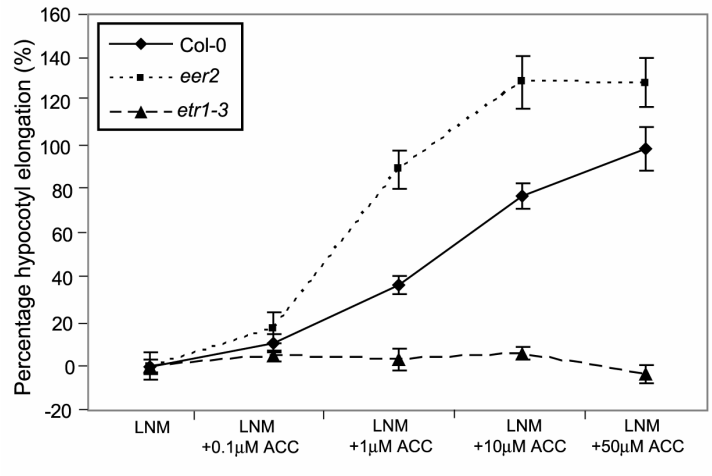

Figure 1: Effect of ACC on hypocotyl elongation in the light (% relative to zero control). Seedlings of wild type (Col-0), *eer2* and *etr1-3* were grown for 8 days on LNM medium in the light supplemented with ACC in a range of concentrations. Data are mean ± SD (n>50).

of 1µM ACC in comparison to seedlings grown in the absence of ACC, whereas only a limited hypocotyl elongation was seen for the wild type under the same conditions (Fig.1). Furthermore, the hypocotyl elongation was significantly more pronounced than that of the wild type on media containing 10µM and 50µM ACC. As expected, the ethylene insensitive mutant *etr1-3* displayed no elongation at all. From these data it was concluded that *eer2* is more sensitive to ACC than the wild type. It should be mentioned that the hypocotyl of *eer2* was longer than that of the wild type even in the absence of exogenously applied ACC. However, as described later, treatment with aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, did not fully revert *eer2* to wild type, indicating that this phenotype of *eer2* is either caused by a growth defect unrelated to ethylene or due to incomplete inhibition at the concentration of AVG used. Another cause for the longer hypocotyl could be that *eer2* is more sensitive to endogenous ethylene.

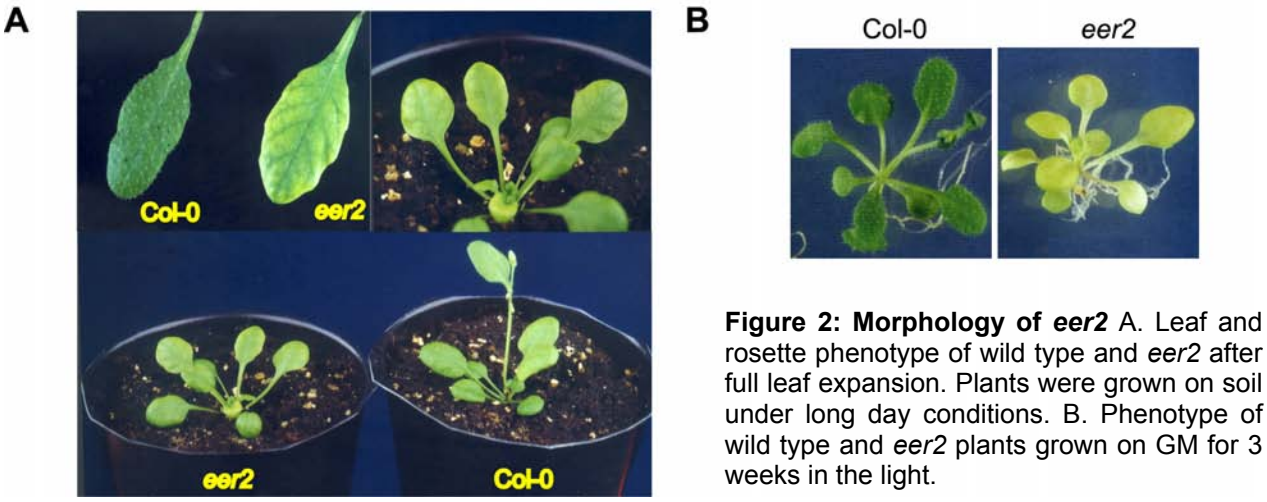

Figure 2: Morphology of *eer2* A. Leaf and rosette phenotype of wild type and *eer2* after full leaf expansion. Plants were grown on soil under long day conditions. B. Phenotype of wild type and *eer2* plants grown on GM for 3 weeks in the light.

The *eer2* mutant was backcrossed to Ler and Col-0. The F1 progeny of both crosses displayed WT-phenotype, while the F2 segregated in a 3:1 ratio, suggesting that the *eer2* mutation is caused by a single recessive allele.

The phenotype of 6 weeks-old *eer2* on soil can be seen in figure 2A. The most striking aspect of this phenotype is the pale color of the interveinal regions of the leaf, resulting in a dark green veination pattern. *eer2* also showed a delay in bolting and flowering time compared to Col-0 wild type (Table 1). In addition, at bolting the number of leaves of the rosette was higher for *eer2. eer2* was in general smaller than wild type, characterized by a smaller rosette and inflorescence stem. Furthermore, measurement of the areas of leaf 5 and leaf 8 of both wild type and *eer2* revealed that leaf 8 of *eer2* is significantly smaller than the wild type leaves. Other characteristics were comparable (Table 1). Interestingly, *eer2* displayed a yellowish phenotype after three weeks on Germination Medium (GM) (Figure 2B). After transfer on soil, the plants turned green again.

Table 1: Biometric analysis of *eer2* relative to wild type.
Values are mean ± SD (n=40).

Assay	Wild type	*eer2*
bolting time (x days after sowing)	45 ± 5	79 ± 8
flowering time (x days after sowing)	53 ± 9	91 ± 9
diameter rozet (cm)	6.2 ± 0.8	5 ± 0.7
# of leaves during flowering	23 ± 4	42 ± 7
length main inflorescence (cm)	33.4 ± 7.8	25.7 ± 6.9
apical dominance (measured by counting the branching of the main inflorescence)	3 ± 0.98	5 ± 2
number of siliques	31 ± 10	26 ± 9
# of seeds/silique	22.8 ± 5.6	24.7 ± 4.3
area of leaves (mm^2) (at time of bolting):		
- leaf 8	63.4 ± 29.4	27.6 ± 6.3
- leaf 5	23.8 ± 11.2	17.2 ±5.2
circumference of leaves (mm) (at time of bolting):		
- leaf 8	32.9 ± 7.2	20.9 ± 2.2
- leaf 5	20.2 ± 4.2	16.1 ± 2.6

5.3.2. Enhanced ethylene response of *eer2* is specific to the hypocotyls in the light

Further experiments on *eer2* demonstrated that the hypersensitive effect is specific for the hypocotyls and that ethylene induced an inverse effect in roots. The root growth inhibition on LNM in the light was significantly lower than in wild type, while absolute root length on

LNM was similar to wild type. Figure 3 shows more than 50% inhibition of root growth in the presence of 0.1 μM ACC for wild type, whereas the same response is only seen in the presence of 10 μM ACC for *eer2*. In the presence of higher ACC concentrations the difference in root length between *eer2* and wild type is decreased, indicating that higher ACC concentrations probably overcome the more insensitive response of *eer2.*

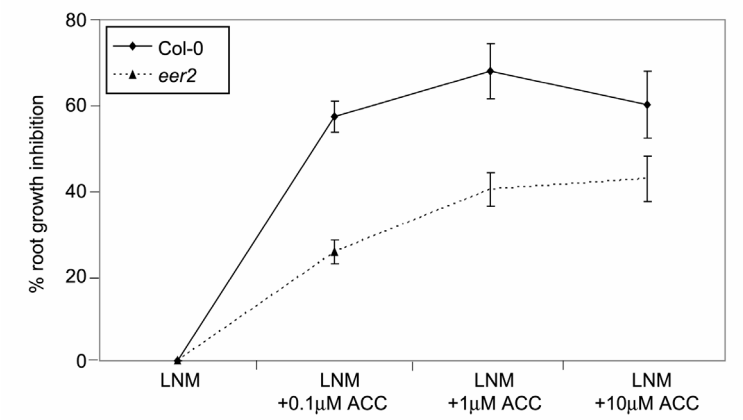

Figure 3: Effect of ACC on root growth in the light (% relative to zero control). Seedlings of wild type (Col-0) and *eer2* were grown for 8 days on LNM medium in the light supplemented with ACC in a range of concentrations. Values are mean ± SD (n=40).

In addition, we measured the shortening of the hypocotyls and root of dark-grown seedlings upon exposure to ACC. This is one of the hallmarks of the triple response. The ethylene-response of hypocotyls and roots grown in the dark on GM was similar to the wild type (Fig. 4A). The insensitive mutants *etr1-3* and *ein2-1,* and the constitutive mutant *ctr1-1* were included and showed the expected insensitive and constitutive behaviour respectively. To

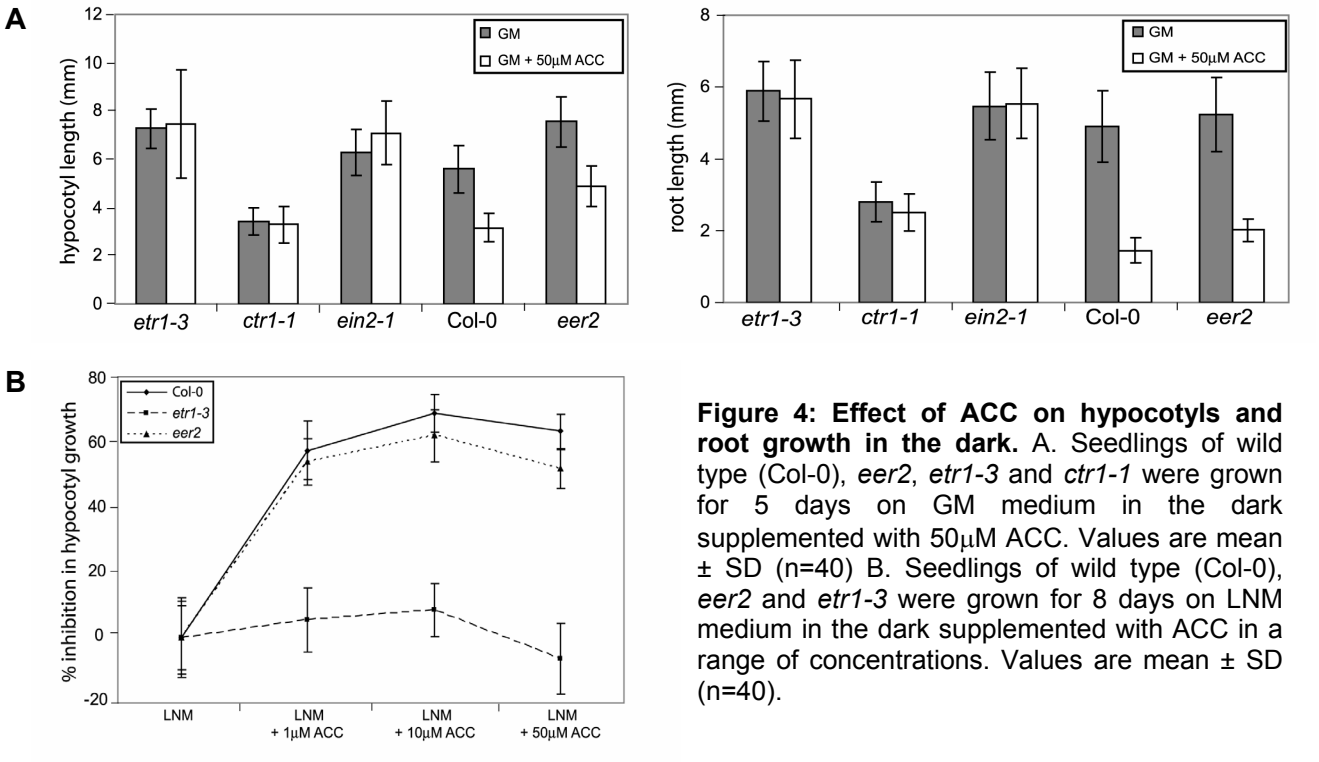

Figure 4: Effect of ACC on hypocotyls and root growth in the dark. A. Seedlings of wild type (Col-0), *eer2*, *etr1-3* and *ctr1-1* were grown for 5 days on GM medium in the dark supplemented with 50μM ACC. Values are mean ± SD (n=40) B. Seedlings of wild type (Col-0), *eer2* and *etr1-3* were grown for 8 days on LNM medium in the dark supplemented with ACC in a range of concentrations. Values are mean ± SD (n=40).

determine if the medium composition could have influenced the result, we also tested the inhibition of hypocotyl length on LNM in the dark (Fig.4B). Here again, *eer2* and wild type responded similar to ACC, thus the medium has no impact on the phenotype. From these results we can conclude that the hypersensitive response is specific to the hypocotyls and specifically affected in the light at the seedling stage in *eer2.*

5.3.3 Epinastic response of *eer2*

Hypersensitivity to ACC is also reflected by the epinastic response of the cotyledons. This response is a known ethylene response and is clearly seen in the *ctr*-mutant. In each panel in figure 5, the seedlings on the left are Col-0, the ones on the right hand are *eer2* seedlings, grown on LNM in the light. In the presence of 1μM ACC, epinasticity of the cotyledons is clearly visible in *eer2*, while in wild type at least 10μM ACC is necessary and the response is not as strong as in *eer2.* The same was observed at higher ACC concentrations. A similar difference in response was seen in the first leaf pair. Furthermore, this response was alleviated by MCP-treatment, even in the presence of 50 μM ACC (data not shown).

Figure 5: Epinastic response of *eer2*. Seedlings of wild type (left) and *eer2* (right) were grown for 8 days on LNM medium in the light supplemented with ACC in a range of concentrations.

5.3.4 Is *eer2* affected in ethylene signalling?

To demonstrate a true ethylene dependent nature of the *eer2* mutation, treatment with 1-methylcyclopropene (1-MCP), which is a competitive inhibitor of ethylene signalling, was performed. As presented in figure 6A, addition of 250ppm MCP could block the hypocotyl elongation in wild type and *eer2.* The disappearance of the hypersensitive response of *eer2* in the presence of ACC and MCP supports the fact that the ACC effect was ethylene-

dependent. Differences in absolute values between figure 6 and figure 1 are probably due to the different growth conditions (see experimental procedures).

In a complementary experiment, 1μM AVG, an inhibitor of ethylene biosynthesis, was added. On medium containing AVG, *eer2* hypocotyls displayed a slightly stronger decrease in hypocotyl length compared to wild type (Fig. 6B). The same response was observed for two other inhibitors of ethylene biosynthesis, AIB (aminoisobutyric acid) and Co^{2+} (data not shown). However *eer2* hypocotyls were still longer than WT on LNM+AVG, indicating that this phenotype of *eer2* is probably ethylene-independent. In addition, when treated with 100μM ACC in the presence of AVG, *eer2* seedlings still showed a more pronounced response to ACC in comparison to WT. The response of the *eer2* roots to AVG, was similar to wild type (data not shown).

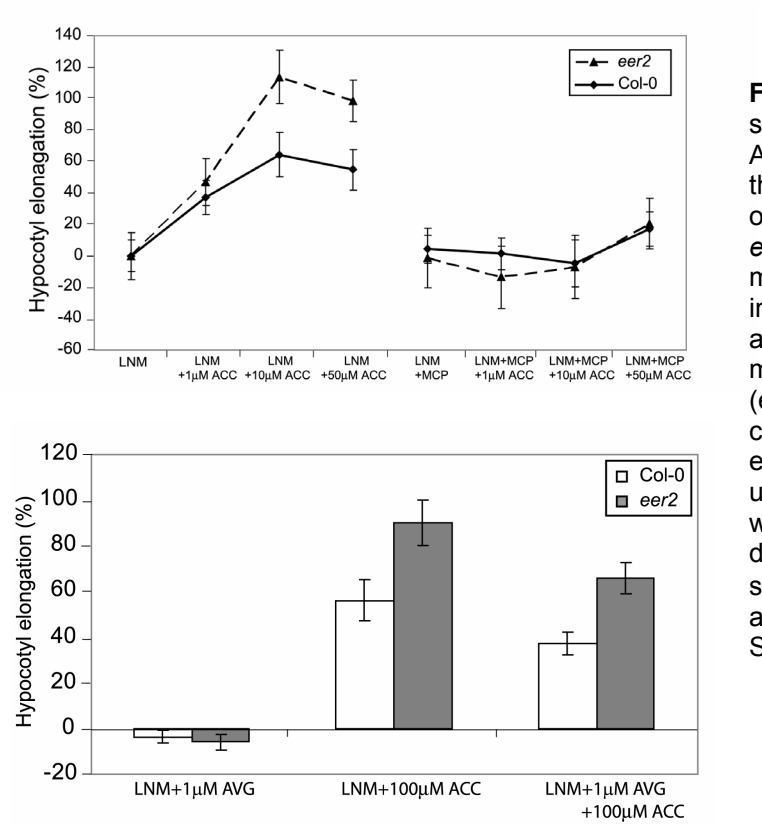

Figure 6: A. Effect of ACC, MCP (ethylene signalling inhibitor) and the combination ACC and MCP on hypocotyl elongation in the light (% relative to untreated seedlings on LNM). Seedlings of wild type (Col-0) and *eer2* were grown for 8 days on LNM medium in the light supplemented with ACC in a range of concentrations, 250ppm MCP and the combination thereof. Values are mean ± SD (n=40) B. Effect of ACC, AVG (ethylene biosynthesis inhibitor) and the combination of ACC and AVG on hypocotyl elongation in the light (% relative to the untreated controls on LNM). Seedlings of wild type (Col-0) and *eer2* were grown for 8 days on LNM medium in the light supplemented with 100μM ACC, 1μM AVG and the combination. Values are mean ± SD (n=40).

Measurements of ethylene released by *eer2* were also performed. Plants were grown for 3 weeks on GM. *eer2* and wild type plants accumulated a similar level of ethylene (Table 2). Thus, ethylene overproduction is probably not the primary cause of the *eer2*-phenotype.

Table 2: Ethylene production by 3 weeks old plants

Strain	Ethylene production (ng ethylene/h/g)
Col-0	2.4 ± 0.2
eer2	2.1 ± 0.3

Plants were grown for 3 weeks on GM. Mean ± SE values were determined from six samples. Ethylene was collected after a period of 2 days.

5.3.5 The *eer2*-mutation influences ethylene-regulated gene expression

To investigate whether the hypersensitivity to ethylene in *eer2* was also reflected at the molecular level, the expression of an ethylene-regulated gene, *GST2,* gluthatione-S-transferase, was analyzed (Zhou and Goldsbrough, 1993). Northern blot analysis was carried out using total RNA isolated from 19-days old plants grown on GM, treated for 24 hours with purified air and 1ppm ethylene (Fig. 7). In *eer2* the *GST2* expression highly exceeded the level of WT-control treated with purified air, a feature reminiscent of *ctr1-1.* In wild-type plants the steady state mRNA-level for *GST2* increased upon treatment with ethylene. However, the *GST2*-transcript was not significantly induced in *eer2* after treatment. The two ethylene-insensitive controls, *etr1-3* and *ein2-1*, displayed no induction in *GST2*-expression. The response of the *ctr1-1* mutant to ethylene is noteworthy. Ethylene responsiveness of three *ctr1* mutants was also found in terms of hypocotyl shortening and increased radial thickness (Larsen and Chang, 2001).

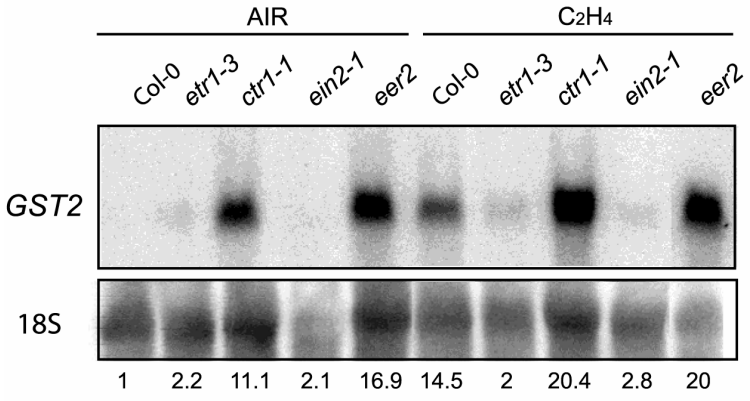

Figure 7: Analysis of *GST2* gene expression. Wild type, *etr1-3*, *ctr1-1*, *ein2-1*, and *eer2* plants were grown for 19 days on GM medium. These plants were exposed to either purified air or 1ppm ethylene for 24 hours. Northern blot analysis was performed using 30 μg of total RNA. 18S rRNA was used as loading control. The indicated numbers represent the normalized signal intensities relative to wild type control.

5.3.6 *eer2* does not senesce faster than wild type

Given the chlorotic nature of *eer2* on GM and soil (Fig.2) and the described role of ethylene in senescence, we examined the senescence pattern of *eer2*. To that end, the total chlorophyll content in excised leaves was measured. Four weeks old *eer2* plants (starting point), grown on soil, showed significantly lower chlorophyll content than the wild type (Fig. 8A). At day 32 after the start of measurements, chlorophyll was not measurable anymore in wild type. In contrast, *eer2* retained approximately 60% of total chlorophyll content relative to the starting point. At day 36, mutant leaves still contained a significant amount of chlorophyll. From these data, it can be concluded that the breakdown of total chlorophyll in *eer2* is much slower than in the wild type (Fig. 8B). This implies that leaf senescence does not occur earlier in *eer2.* In addition, the chlorophyll loss is even slower

in *eer2* than in both insensitive control mutants, for which a delay in senescence is described (Fig. 8B).

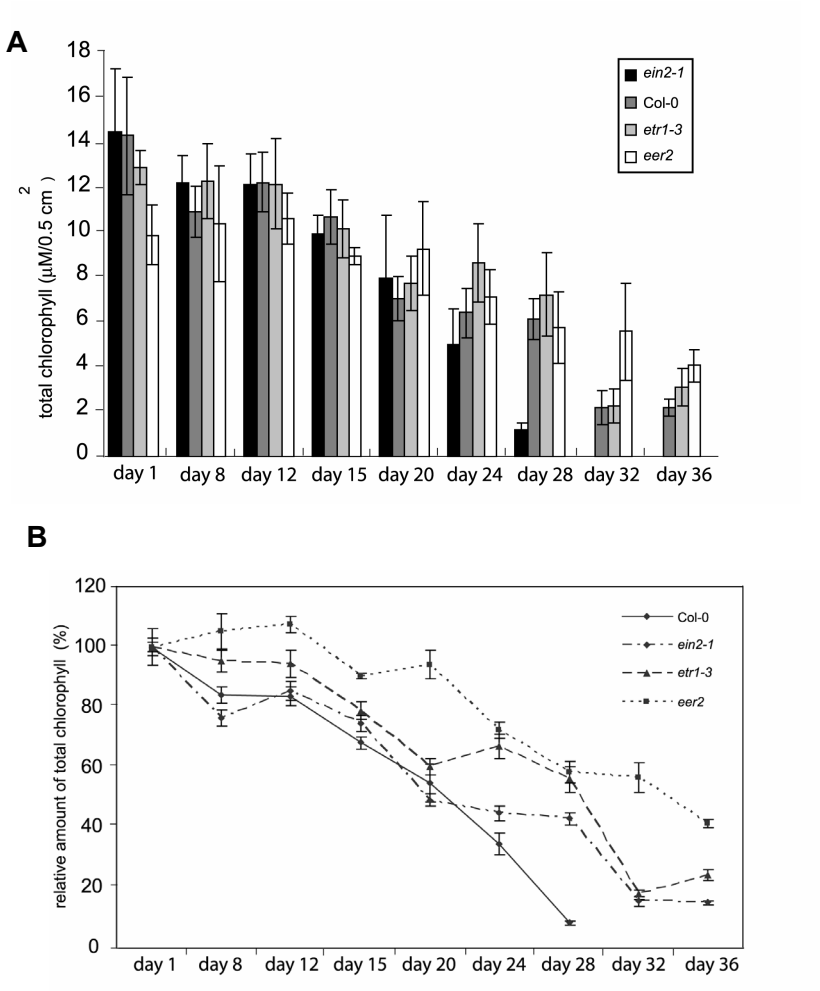

Figure 8: A. Chlorophyll content during rosette development of wild type, *eer2*, *ein2-1* and *etr1-3*. Leaf disks of full-grown rosette leaves 5 and 6 were harvested after 4 weeks of growth (day 1) and subsequently 8, 12, 15, 20, 24, 28, 32, and 36 days later. Results of chlorophyll content are shown as mean of 5 replicates. Error bars represent SE. B. Total chlorophyll amount relative to day 1 for wild type and mutants.

In parallel, an additional assay to quantify only photosynthetically active chlorophyll was performed. The method is based on an in vivo measurement of chlorophyll fluorescence, represented by the overall quantum yield of photochemical energy conversion (Fm'-Ft)/Fm' (see materials and methods) (Lootens and Vandecasteele, 2000). The analysis of chlorophyll fluorescence yielded similar results as the spectrophotometric measurement of total intact chlorophyll molecules for wild type and *eer2* (Fig. 9).

A large collection of reticulate leaf mutants is available in *Arabidopsis* stock centers. We analysed the sensitivity to ACC on LNM in the light and the phenotype on GM in the light for the *arc* (accumulation and replication of chloroplasts) mutants and the *ch* (chlorina) mutants (data not shown). None of these mutants displayed the characteristics of *eer2*, indicating that probably none of them are allelic to *eer2*.

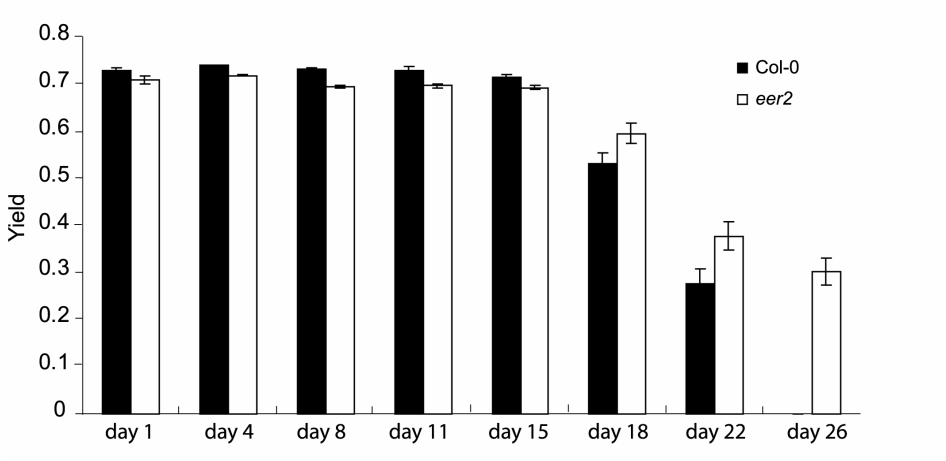

Figure 9: The effective quantum yield of photochemical energy consumption, ΔF/Fm' (Y) (=yield) was quantified *in vivo* after 4 weeks of growth (day1) on soil and subsequently 4, 8, 11, 15, 18, 22, and 26 days later. Results are shown as mean of 30 replicates. Error bars represent SE.

5.3.7 Supplementation of Hoagland with Zn^{2+} or Mn^{2+} reduces leaf chlorophyll contents in *eer2*

By growing the *eer2*-mutant on both GM and Hoagland, we noticed a striking difference. On GM *eer2* showed a completely chlorophyll loss after 3 weeks resulting in yellow leaves. Surprisingly this process didn't happen when growing *eer2* in Hoagland (Fig. 10A). To understand this phenomenon, a comparison of both media was made in order to identify which factor causes this difference in phenotype. Since Hoagland only contains the mineral salts (Table 3A), we removed those ingredients that are specific for GM one by one (see table 3B). *eer2*-plants grown on GM-media without myoinositol, without vitamines or without sugar still resulted in yellow plants. These results inferred an implication of mineral salts in the conspicuous phenotype of *eer2* on GM. We calculated the absolute concentrations of the cations and anions present in GM and Hoagland. Adjusting the concentrations of Mn^{2+} and Zn^{2+} in Hoagland to those amounts present in GM, resulted in interesting observations. 3-weeks old mutant plants grown on Hoagland-medium supplemented with additional Mn^{2+} displayed a yellow phenotype (Fig. 10B). The same happened on Hoagland-medium supplemented with additional Zn^{2+} (Fig. 11)· In both cases, especially in the presence of Zn^{2+}, the plants stayed smaller than the control plant. This can possibly be explained by the fact that the Mn^{2+}-concentration is increased 10 times and the Zn^{2+}-concentration about 200 times. The plants could sustain these high amounts of Mn^{2+} and Zn^{2+} because they fully recovered totally on soil.

Table 3A: Overview of ingredients and minerals in GM and Hoagland

GM	Hoagland
Vitamins:	/
thiamin (1mg/l)	
pyridoxin (0.5mg/l)	
nicotinic acid (0.5mg/l)	
sucrose (10g/l)	/
myo-inositol (100mg/l)	/
MES (0.5g/l)	/
Murashige and Skoog salt mixture:	
Ca^{2+} :158,9 mg/l	Ca^{2+} :40 mg/l
Cl^{-} : 281,1 mg/l	Cl^{-} : 78,1 mg/l
Co^{2+} :0,01 mg/l	/
Cu^{2+} :0,001 mg/l	Cu^{2+} : 0,015 mg/l
SO_4^{2-} : 314,6 mg/l	SO_4^{2-} : 327,5 mg/l
H^{+} :2,8 mg/l	H^{+} :0,90 mg/l
BO_3^{3-} : 5,9 mg/l	BO_3^{3-} : 1,11 mg/l
K^{+} :783,8 mg/l	K^{+} :40,1 mg/l
PO_4^{3-} : 118,6 mg/l	PO_4^{3-} : 39,9 mg/l
I^{-} : 0,6 mg/l	/
Mg^{2+} :74,7 mg/l	Mg^{2+} :81,8 mg/l
Mn^{2+} :8,1 mg/l	Mn^{2+} :0,8 mg/l
Na^{+} :2,4 mg/l	Na^{+} :9,7 mg/l
MO_6^{-} : 0,1 mg/l	MO_6^{-} : 0,05 mg/l
NH_4^{+} :371,3 mg/l	NH_4^{+} :25,7 mg/l
NO_3^{-} : 2444 mg/l	NO_3^{-} : 88,5 mg/l
Zn^{2+} :3,8 mg/l	Zn^{2+} :0,02 mg/l
Fe^{3+} :5,6 mg/l	Fe^{3+} :3,3 mg/l
EDTA :28,8 mg/l	/

Table 3B: Overview of phenotypes of Col-0 and *eer2* on different media

	phenotype of Col-0 on	phenotype of *eer2* on
GM - vitamines	green	yellow
GM - sucrose	green	yellow
GM - myo-inositol	green	yellow
GM - MS	no viable plant	no viable plant
Hoagland + $CuSO_4$	green	green
Hoagland + NH_4NO_3	green	green
Hoagland + K_2SO_4	green	green
Hoagland + H_3BO_3	green	green
Hoagland + $MgSO_4$	green	green
Hoagland + $CaCl_2$	green	green
Hoagland + $ZnSO_4$	green	yellow
Hoagland + $MnCl_2$	green	yellow

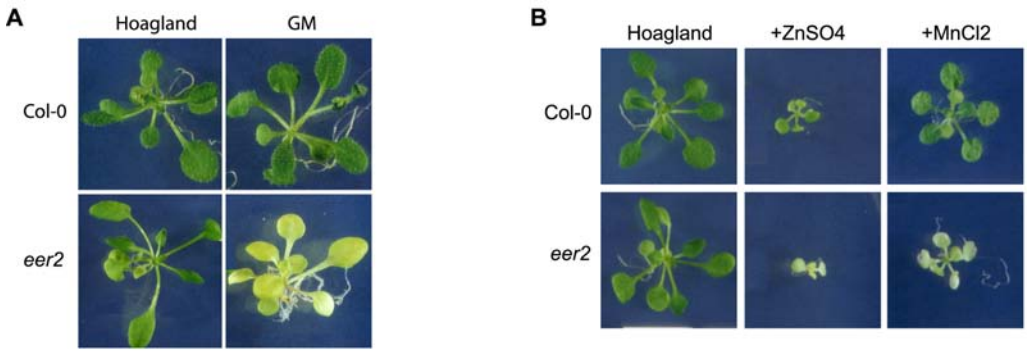

Figure 10: A. Phenotype of 3 weeks old wild type and *eer2* grown on Hoagland medium (left panels) and GM medium (right panels) B. Phenotype of 3 weeks old wild type (upper panels) and *eer2* (lower panels) grown on Hoagland medium, Hoagland supplemented with $ZnSO_4$, and Hoagland supplemented with $MnCl_2$.

Due to the paler phenotype of *eer2* on GM compared to that on Hoagland, we investigated the amount of *CAB*-mRNA (chlorophyll a/b binding protein) in *eer2* on both media. Corresponding to the phenotypic results, the *CAB*-expression was dramatically decreased in 3 weeks old *eer2* plants (see figure 11) on GM, whereas the expression was slightly higher in *eer2* on Hoagland as compared to the wild type.

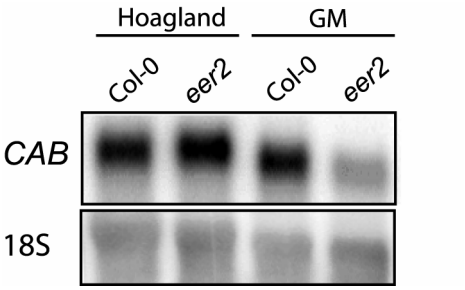

Figure 11: Analysis of *CAB* gene expression. Wild type and *eer2* plants were grown for 3 weeks on Hoagland and GM medium. Northern blot analysis was performed using 25 μg of total RNA. 18s rRNA was used as loading control.

5.3.8 Response of *eer2* to auxin and trans-zeatin.

Previous research demonstrated that mutations in ethylene signalling components often conferred changes in sensitivity to other hormones as well (Lehman et al., 1996; Vogel et al., 1998). Therefore we analysed the response of *eer2* to cytokinin and auxin. A dose-response experiment for zeatin was set up for the measurement of the root length inhibition in WT and *eer2*. Trans-zeatin is a cytokinin and cytokinins are known to induce ethylene biosynthesis. Therefore the inhibition of rootlength can be considered as a result of cytokinin-induced ethylene production. Wild type and *eer2* seedlings displayed a similar response to the range of concentrations of zeatin (data not shown).

Also auxin has been reported to stimulate ethylene production (Rodrigues-Pousada et al., 1993). Nevertheless, Vandenbussche *et al.* suggested that the ethylene effect on

hypocotyl elongation on LNM in the light is mediated through auxins (Vandenbussche et al., 2003). Thus, the latter act downstream of ethylene in the elongation process To test the response of *eer2* to auxin, we treated wild type, control mutants and *eer2* with 6μm IAA and with the combination of 6 μm IAA and an auxin transport inhibitor TIBA. The wild type and the insensitive mutants displayed comparable elongation, whereas *ctr1-1* showed no significant difference in hypocotyl length (Figure 12). This is corresponding to the results of Vandenbussche et al. (2003). In addition, no differences between wild type and the *eer2*

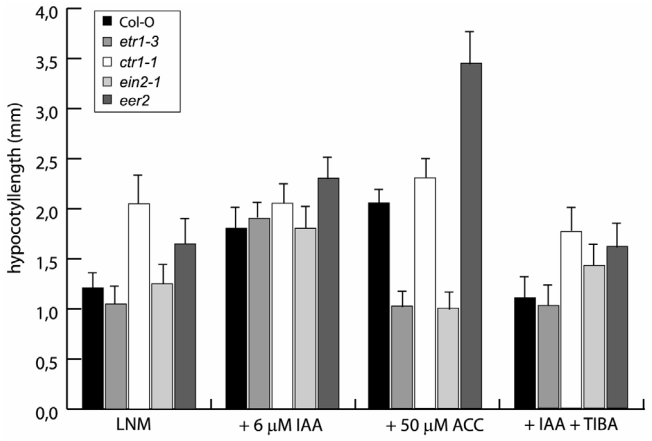

Figure 12: Effect of IAA, ACC, and IAA+TIBA on hypocotyl elongation in the light in Col-0, *etr1-3*, *ctr1-1*, *ein2-1*, and *eer2*. Seedlings were grown for 8 days on LNM medium in the light supplemented with the indicated concentration of IAA, ACC, and IAA+TIBA in a long day photoperiod. Values are mean ± SD (n=40).

mutant were observed, demonstrating that auxin signalling is not affected in *eer2* at this stage of development under the conditions used. Moreover, the combination TIBA/IAA in the growth medium also yielded similar responses in wild type and *eer2* and the hypersensitive response was only seen in the presence of ACC*,* further indicating that this response is specific to ethylene signalling at this stage of development.

5.3.9 Map location of the *eer2* locus

Using a cross between *eer2* and wild type Landsberg, SSLP and AFLP mapping were performed. *EER2* was linked to the top arm of chromosome 4 and to the center of chromosome 1. For chromosome 4, the *eer2* phenotype showed linkage with BA-12L (15.2cM) and nga8 (26.56 cM). For chromosome1, linkage analysis placed the *EER2* locus at the position of markers SM19_96,2 (60 cM) and SM19_106,4 (68 cM). This result demonstrated that the FN-mutagenized *eer2* mutant is probably the result of a chromosomal rearrangement. Indeed, by checking the segregation results of the F2-generation of the crosses between Ler x *eer2* (35 *eer2*: 126 WT) and Col-0 x *eer2* (18 eer2: 69 WT) in more detail, approximately one fifth of each population displayed the *eer2*-phenotype, indicating a rearrangement between chromosomes took place in *eer2*

(personal communication by M. Vuylsteke). Detailed results of the linkage analysis for all individual samples on both chromosomes are supplemented as additional data (5.7, page 148). In addition, an overview of this analysis is presented on pages 138 and 139.

5.4 DISCUSSION

In this report, we have described the initial characterization of a new recessive *Arabidopsis* mutant showing a hypersensitive response to ethylene. This hypersensitive response was specific to the hypocotyls grown on LNM in the light. Under these conditions, the hypocotyls length of *eer2* were doubled in length at 1μM ACC, whereas the wild type required more than 10 μM ACC to achieve the same level of elongation. Roots of *eer2* were slightly less sensitive than the wild type to low concentrations of ACC.

There are several lines of evidence indicating that the *eer2* mutant phenotype results from a defect in the ethylene response. The alleviation of the hypersensitive response and the epinastic cotyledons by the ethylene antagonist 1-MCP shows that the ACC-effect is a true ethylene effect and that the *eer2* phenotype is ethylene-dependent. In addition, in the presence of ACC and the ethylene biosynthesis inhibitor AVG, lowering the endogenous ethylene production, the hypocotyls were smaller than when ACC alone was added, further indicating that the hypersensitive response is ethylene dependent.

Similar phenotypes were described previously for the *eer1* mutant (Larsen and Chang, 2001). This mutant displayed enhanced ethylene responsiveness in both the hypocotyl of dark-grown seedlings and adult inflorescence stems. Molecular cloning of the *eer1* mutation revealed that it is a new allele of *RCN1*, an A regulatory subunit of PP2A (Larsen and Cancel, 2003). *eer1* maps on chromosome 1 (between CAPS marker m235 and *unusual floral organs* (*ufo*). In contrast to *eer1*, the enhanced ethylene response in the hypocotyl of *eer2* was only seen in the light and not in the dark, which underlines the importance of new screening methods. Moreover, *eer1* overproduces ethylene, which is not the case for *eer2*, indicating that these mutants are probably not allelic. Further evidence that *eer2* seems to be a new locus in ethylene signalling, was given by the map location, which is significantly closer to the centromere of the chromosome 1 than *eer1*.

As described, ethylene-induced hypocotyl elongation of light-grown seedlings is likely to be transduced by the same pathway as the one that controls inhibition of hypocotyl elongation in the dark (Smalle et al., 1997). The opposite effects suggest the involvement of light-

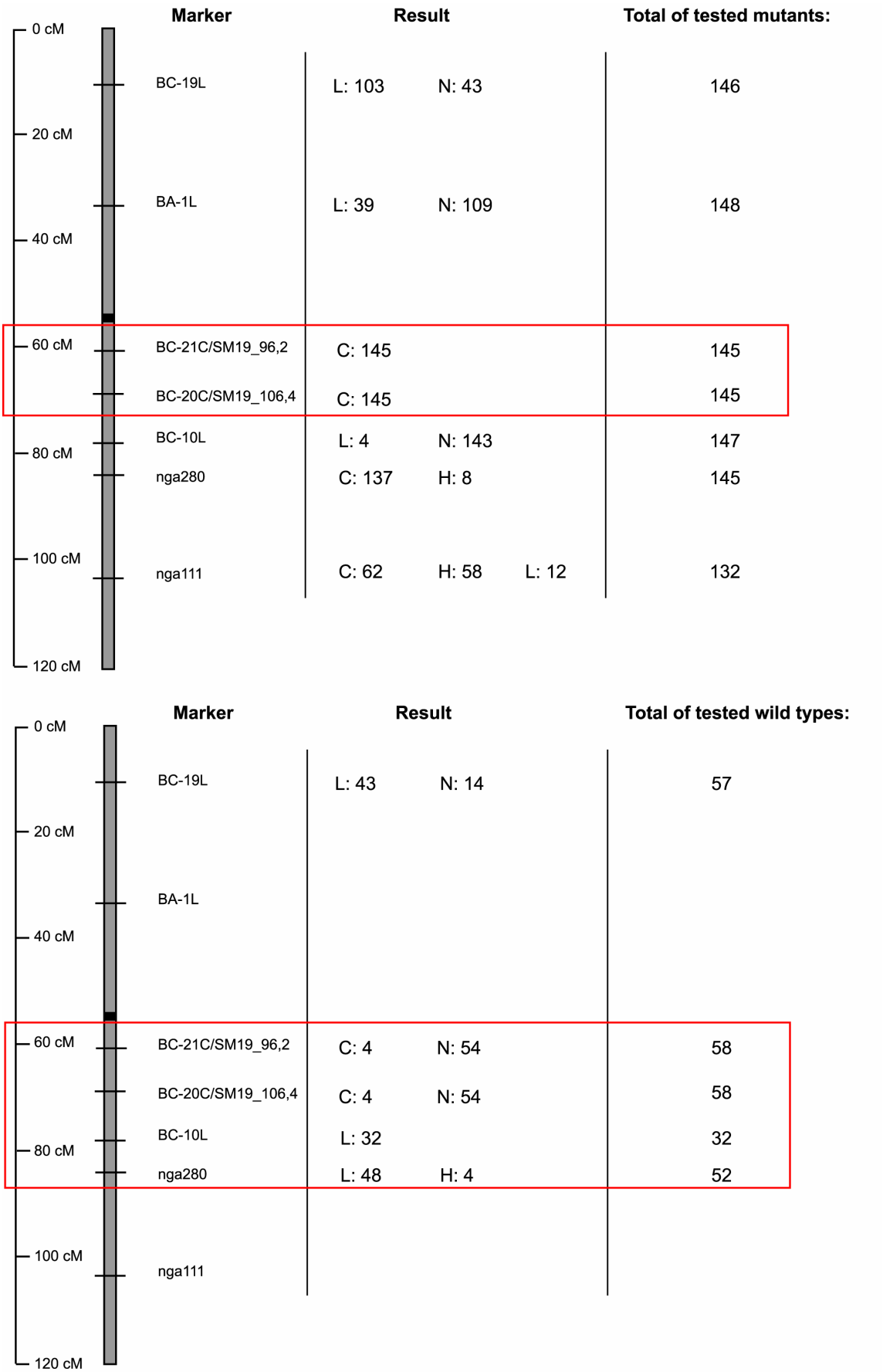

Figure 13: Results of the AFLP-and SSLP-mapping for markers on chromosome 1. Boxed markers indicate the place of highest linkage; within this box the position of *EER1* is expected. L: Landsberg band, C: Columbia band, H: heterozygous band, N: no band

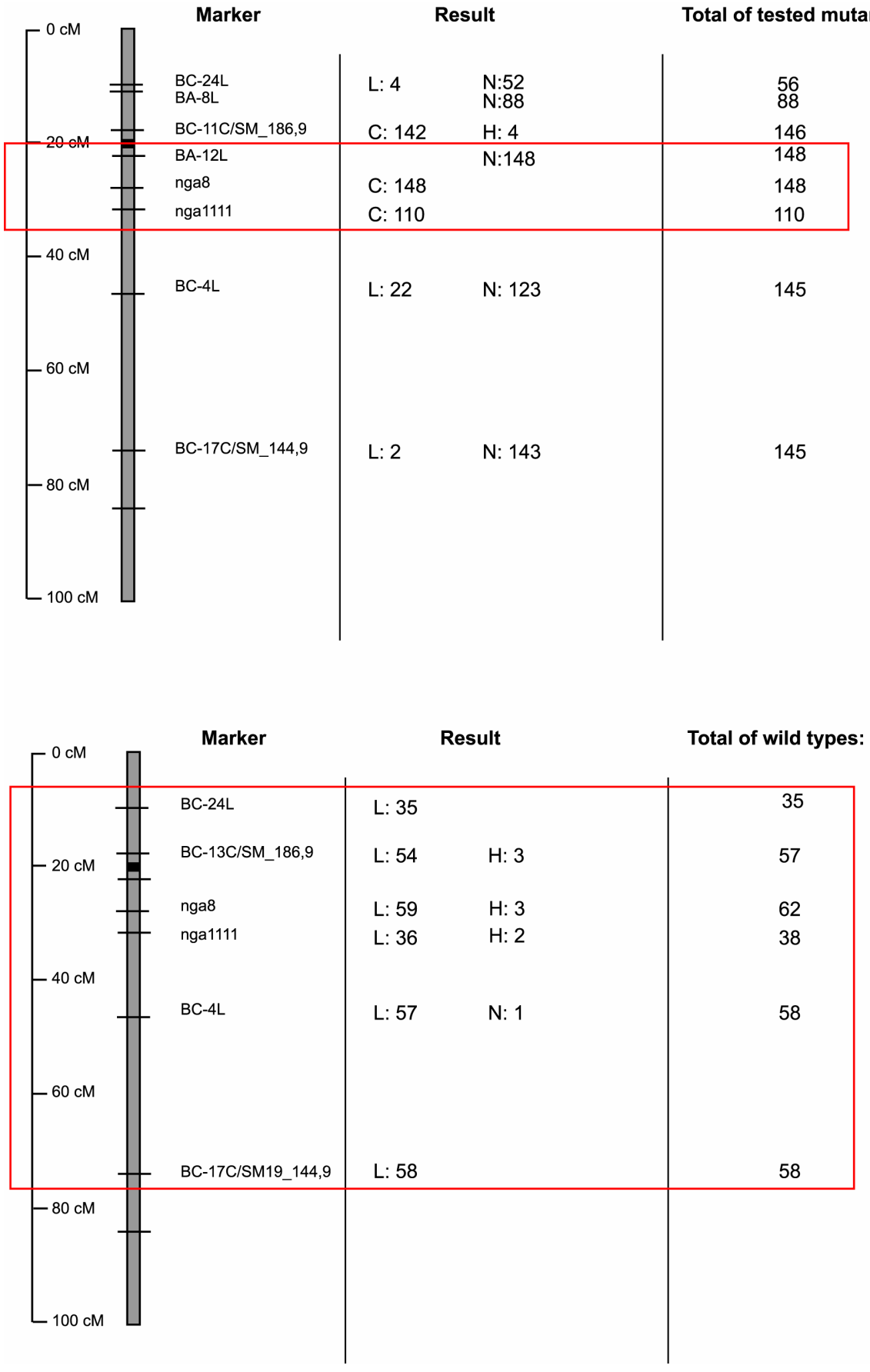

Figure 14: Results of the AFLP-and SSLP-mapping for markers on chromosome 4. Boxed markers indicate the place of highest linkage; within this box the position of *EER1* is expected. L: Landsberg band, C: Columbia band, H: heterozygous band, N: no band

regulated upstream or downstream components. Since the hypersensitivity towards ethylene is not seen in the dark, the *EER2* gene could be a component involved in this ethylene-stimulated hypocotyl elongation in the light. In this case, the *EER2* product is proposed to negatively regulate the ethylene signal in certain ethylene responses in the light and may have an opposite function in roots.

5.4.1 A role for *eer2* in senescence?

At later stages of development *eer2* was still characterized by enhanced ethylene sensitivity. Cotyledons and the first leaf pair were epinastic and *GST2,* a known ethylene responsive gene, was highly expressed in untreated 19-days old *eer2* plants grown on GM. Plant GSTs have well described roles in the detoxification and tolerance of crops to herbicides (Lamoureux and Rusness, 1993). In addition, plant GSTs can also act as glutathione peroxidases (Bartling et al., 1993; Edwards et al., 2000). A regulation of ethylene-induced GSTs during senescence is discribed (Itzhaki et al., 1994). Interestingly, the *eer2* plants were totally yellow when grown on GM medium. In *Arabidopsis*, visible yellowing is widely used to stage the progression of senescence and correlates with other biochemical changes that occur during leaf senescence (Hensel et al., 1993; Lohman et al., 1994). Therefore the high accumulation of GST2 in *eer2* in the absence of ethylene could be linked with the altered senescence pattern in *eer2*. In this view, also the lower expression level of *CAB*, a marker for the photosynthetic stage of development, can be explained. Previously, it was shown that leaf senescence is triggered by an age-related decline in photosynthetic processes (Hensel et al., 1993). Ethylene plays an important role in leaf senescence. Genetic evidence for this is given by the *etr1* and the *ein2* mutant which both have a delayed senescence (Bleecker et al., 1988; Grbic and Bleecker, 1995; Chao et al., 1997). Furthermore, two of the four *ore* (oresara) mutants, showing a delayed senescence pattern, are alleles of the *ein2* mutant (Oh et al., 1997). On the other hand, ethylene is neither necessary nor sufficient for the occurrence of senescence. Senescence eventually occurs in the ethylene insensitive mutants and the ethylene constitutive response and overproduction mutants do not show premature leaf senescence. Therefore, ethylene does not directly regulate the onset of leaf senescence; but rather acts to modulate the timing of leaf senescence. Jing et al. further demonstrated that ethylene promotes senescence within a specific age window (Jing et al., 2002). On soil, the chlorophyll content of *eer2* was significantly lower than wild type at 4 weeks of age, but the senescence process was progressing much slower than observed in the WT, even slower

than in *etr1-3* and *ein2-1*. These results suggest that EER2 could be a regulator of the senescence program such that loss-of-function of EER2 provokes precocious age-related signals suppressing photosynthetic-associated genes at the moment that the leaf is not yet primed for senescence. Therefore in the phase prior to the onset of leaf senescence ethylene cannot not promote leaf senescence in *eer2* (similar to a constitutive ethylene mutant).

Although the onset of some hallmarks of senescence are clearly earlier in *eer2* compared to the wild type, the time frame of the senescence process is much wider in *eer2*, indicating that EER2 could also be involved in self-maintenance. The existence of genes involved in retarding the senescence program or in self-maintenance has been described. Mutants for these genes showed earlier senescence. These are for example *hys1* (hypersenescence)/cpr5 (constitutive pathogene response) (Yoshida et al., 2002), the *at2-mmp-1* mutant (matrix metalloproteinase) (Golldack et al., 2002) , *fiw* (fireworks) (Nakamura et al., 2000) the *t365* mutant (in which the PEAMT (phosphoethanolamine N-methyltransferase) -gene is silenced) (Mou et al., 2002), and the three *old* mutants (onset of leaf death) (Jing et al., 2002). For this last group of mutants the interaction between leaf age and ethylene was investigated. Their analysis indicated that in *old2* and *old1* leaf senescence is dependent on ethylene whereas it is not in *old3*.

The typical phenotype of *eer2* and the lower total chlorophyll content of 4 weeks old plants grown on soil also suggest that the locus possibly may encode a product that functions in chloroplast development or activity. In the case of the *ore4-1* mutant, a T-DNA insertion mutant in the plastid ribosomal small subunit protein 7 (PRPS17), the extended leaf longevity is explained by the reduced functioning of the chloroplast consistent with reduced growth (Woo et al., 2002). Also *eer2* displays both lower chlorophyll content after full leaf expansion and reduced leaf size. Since the chloroplast is the major source of energy input for the plant through photosynthesis, a reduced functioning of the chloroplast would cause a deficiency in energy metabolism. This reduced metabolism could be responsible for the extended longevity in the mutant.

5.4.2 Accumulation or translocation of metals affected in *eer2*?

Another interesting observation was that *eer2* mutants grown on Hoagland-medium for 3 weeks failed to show the yellowish phenotype which was typical for growth on GM-medium. The addition of the divalent cations Zn^{2+} and Mn^{2+} to Hoagland could induce the chlorotic phenotype again. What can we learn from these observations? Zinc is an

essential catalytic component of over 300 enzymes, including alkaline phosphatase, alcohol dehydrogenase, Cu/Zn superoxide dismutase, and carbonic anhydrase. In addition, zinc also plays a critical structural role in many proteins, for example transcription factors. On the other hand, manganese is required for a number of essential processes in plants, including oxygen evolution in photosynthesis, detoxification and CO_2 fixation in C4 and CAM plants. So, both essential nutrients are required for some processes in the chloroplast. Previously, the *man1* mutant (for manganese accumulator) has been described (Delhaize, 1996). This mutant was found to cause *Arabidopsis* seedlings to accumulate a range of metals, indicating that the *man1* mutation disrupts the regulation of metal-ion uptake or homeostasis in *Arabidopsis*. The *man1* mutant was chlorotic, dwarfed and flowered later than the wild type when grown on soil. In addition, toxicity symptoms by high concentrations of Zn^{2+} are also characterised by chlorosis (Macnair et al., 1999). Therefore, one explanation could be that in *eer2* the uptake of external metals or their translocation is hyperactive. Little is known on metal transporters in *Arabidopsis*. Most of the recent studies on metal transport are done by functional complementation tests of yeast mutants disabled in metal-uptake. For Mn^{2+} and Zn^{2+} these studies are respectively done on the *smf1* mutant strain (defect in Mn uptake) and on the *zrt1zrt2 Sacccharomyces cerevisiae* mutant (defect in Zn uptake) (Korshunova et al., 1999). The broad substrate protein IRT1 could complement the *smf1* mutant. This IRT1 protein also complements the *zrt1zrt2* mutant, linking Mn^{2+} and Zn^{2+} nutrition. Interestingly, four close homologues of IRT1 (ZIP1, ZIP2, ZIP3 and ZIP4) in *A. thaliana* have been implicated in the transport of Zn^{2+} (Grotz et al., 1998). Besides the common function in complementing the *zrt1zrt2* mutant, these Zn^{2+} transporters show unique sensitivities to other metal ions that may reflect differences in their substrate specificities. The Zip family is structurally distinct from other metal ion transporters such as the Nramp proteins recently implicated in divalent cation transport (Belouchi et al., 1997). In *Arabidopsis*, functional studies have shown that AtNramp metal transporters modulate Cd and Fe toxicity (Thomine et al., 2000). Interestingly, recent evidence showed that upon Fe starvation, AtNramp3 disruption leads to increased accumulation of Mn and Zn (Thomine et al., 2003). The above-mentioned Smf1 protein from yeast is also a member of the Nramp protein family and functions in the transport of a variety of divalent cations such as Mn^{2+}, Zn^{2+} and Cu^{2+}. Previously, a link between metal ion homeostasis and ethylene signalling has been established. The N-terminal half of EIN2, a central transducer in the ethylene-signaling pathway, has significant homology to the Nramp divalent cation transporters (Alonso et al.,

1999). However, no metal ion transporter activity has been detected for EIN2. Analogies with the yeast glucose sensors led to the proposal that EIN2 might function as a sensor for an upstream signal, presumably divalent cations. Additional evidence for the importance of metals in ethylene-signalling came with the discovery of the copper requirement of ethylene perception (Rodriguez et al., 1999). An interesting result inferring that a transport process is involved in hypocotyl elongation is described by Sidler *et al*. They presented evidence that AtPGP1 (for *Arabidopsis thaliana* P glycoprotein1), belonging to the ABC transporters, is involved in hypocotyl elongation in the light (Sidler et al., 1998).
Future cloning of the *EER2* gene will be the needed and important step to understand the function of the EER2 product in ethylene signalling.

5.5 ACKNOWLEDGEMENTS

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5.6 EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Approximately 33.000 fast neutron (FN) mutagenised M2 Columbia seeds (Lehle seeds) were screened. Columbia (Col-0) was purchased from Lehle seeds (Round Rock, TX). The wild type Ler-0 (Landsberg *erecta*) as well as the ethylene response mutants *etr1-3*, *ctr1-1*, and *ein2-1* originated from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University. The eer2 mutation was backcrossed twice to the Col-0 wild type. Conditions of the growth chamber and greenhouse were 22°C and 60% relative humidity white fluorescent light (75 µmol/m^2 per s) and long day conditions (16 h light/8 h dark).

Media and treatments

The *Arabidopsis* seedlings were grown under sterile conditions as described in Smalle *et al*. (1997). The rich medium used was GM (Growth Medium) supplemented with 0.5 g/L of

MES (Roman *et al.*, 1995). The minerals NH_4NO_3, $CuSO_4$, were obtained from Vel (Leuven, Belgium), $ZnSO_4$, $MnCl_2$, $CaCl_2$ from Sigma-Aldrich (St.-Louis, MO), and K_2SO4, $MgSO_4$, and H_3BO_3 from Merck (Darmstadt, Germany). ACC (1-Amino-Cyclopropane-1-Carboxylic acid), aminoethoxyvinylglycine (AVG), indole-3-acetic acid (IAA), and TIBA (tri-iodo-benzoic acid) were obtained from Sigma-Aldrich (St. Louis, MO). All hormone and inhibitor solutions were added to the medium after filter sterilization. Plates were stored at 4°C in the dark for 2 days and then put in a growth chamber. The MCP was supplied by the Department of Organic Chemistry (Ghent University, Ghent, Belgium). For MCP gassing, seedlings were grown in 30 μmol m^{-2} s^{-1} photosynthetic photon flux density. Treatment with MCP was performed for 20h d^{-1}. Flushing of the growth chamber occurred during the subjective morning for 4h with four refreshments per hour.

Biometric analysis

Measurements of hypocotyl length of light grown seedlings were done as in Smalle et al. (1997) using a Stemi SV 11 microscope equipped with a graduated ocular (Zeiss, Jena, Germany). The rosette diameter was measured after full leaf expansion using a ruler with 1 mm precision. The number of leaves was counted at flowering time (from the moment the first flower was open). Measurement of the inflorescence, branching, pedicule and silique heights as well as the number of siliques and the number of seeds per silique were performed on dry plants. Surface area measurements of leaf 5 and leaf 8 after full leaf expansion were obtained by taping the respective seedlings on Whatmann 3mm paper, followed by scanning and subsequent analysis of the images using Scion Image software (Scion Corporation, Frederick, MD).

Ethylene measurements

For ethylene measurements, wild type and *eer2* seeds were sterilized and sown on GM medium. The seedlings were grown for 19 days, 5-10 seedlings were harvested and capped in a 10 ml vial. Emanation of ethylene was measured after 2 days by gas chromatography (flow-through system) as described by (De Greef et al., 1976). The measurements were repeated three times, each with six samples per line. All measurements were performed at the same time of the day. Settings: inlet: 150˚C, 11,50 psi, 9 ml/min; column: 11,50 psi, 6 ml/min; oven: 180˚C

Ethylene treatments

Wild type (Col-0), *eer2* and the control ethylene response mutants were sown under sterile conditions (Smalle *et al*., 1997) on GM. At 19 days after sowing, the plants were placed inside a chamber dedicated to gas exposures. Subsequently, 1ppm of ethylene or air (organic carbon free, Air Liquide Belge N.V., Aalter, Belgium) were flushed through at a flux rate of 250 mL min^{-1}. Treatment was done for 24hrs followed by Rnase-free harvesting of samples. Experiments were repeated independently 3 times. Ambient conditions were 22 °C, 60% humidity and white fluorescence light (75$\mu M/m^2/s$) under long day conditions (16h light/8h dark).

RNA preparation and gel blot analysis

Total RNA was extracted using Trizol^R reagent (GIBCO/BRL, Gaitherburg, MD) according to the manufacturers instructions. Depending on the experiments, 30μg of total RNA was denaturated and loaded on 1.2% (w/v) agarose gel. Gel- and running buffers contained 10mM MOPS/10mM TEA. The RNAs were blotted to nylon membranes (Hybond N, Amersham, UK) in 25mM phosphate buffer as described (Sambrook et al., 1989). Blotting was followed by baking the filter at 80° for 2h. Hybridization was at 65° following the method of Church and Gilbert (Church and Gilbert, 1984). Probes used in this research were the EcoR1 insert of a cDNA clone encoding an ethylene-regulated glutathione-S-transferase (Zhou and Goldsbrough, 1993), an *Arabidopsis thaliana* 18S ribosomal RNA probe to allow normalisation, and the CAB-probe from *Columbia* wild type made by cloning a CAB PCR-fragment in the pGemT-vector, all 32P labeled. Radiolabeling was performed by random priming and polymerisation using the T7-Quick Prime Set. Subsequently, the membranes were washed with 2xSSC, 0.1% SDS followed by 0.2xSSC, 0.1% SDS. Filters were exposed to Kodak films (XAR) for about 24h. The signals were quantified using the ImageQuant program on a phosphorimager.

Measurement of chlorophyll levels

About 40 WT- and mutant plants were grown on soil in the greenhouse. Samples of leaves 5 and 6 were taken starting from 3 weeks after sowing, with intervals of 5 days. At each

time point the length of leaves was measured and a leaf disk of about 0.5 cm^2 was perforated from the widest part of the leaf blade. For the chlorophyll determination, first all the disks were ground in liquid nitrogen. This was followed by incubating the pulverized tissue in 80% acetone (500ml/0.5cm^2) for 3 hours in the dark. After briefly shaking, the samples were centrifuged for 5'at 5000rpm. The supernatant was measured in the spectrophotometer (DU®-64 Spectrophotometer, Beckman) at 664 and 647 nm. Calculations for the chlorophyll a-, chlorophyll b-, and the total chlorophyll amount was done by following formulas:

Chl (a) (μM)= 13.19. A_{664}-2.57. A_{647}

Chl (b) (μM)= 22.10. A_{647}-5.26. A_{664}

total chl. (μM)= 7.93. A_{664}+19.53. A_{647}

In addition, a portable pulse amplitude modulated fluorometer (PAM-2000; H. Walz) was used to determine effective quantum yield, ΔF/Fm' (Y) in a similar experiment (Lootens and Vandecasteele, 2000). The light-emitting diode (LED) was positioned at a distance of 3 mm above the adaxial front surface. After 0.6 s, a LED light source was switched on. Finally, quantum yield, ΔF/Fm' (Y), was determined during 26 days with a 4 days interval between individual measurements.

Linkage analysis

The increase in hypocotyls length on ACC, the delay in bolting and flowering, and the pale phenotype are traits that cosegregated in a population of 161 F_2 plants of an *eer2* backcross with Col-0 in a recessive fashion (mutant: wild type, 35:126). Mapping of the *eer2* locus was performed with simple sequence length polymorphism markers (Bell and Ecker, 1994) and amplified fragment length polymorphism markers (Vos et al., 1995). *eer2* was crossed to Ler. The F_2 population was scored for mutant and wild type plants. Per F_2 individual, DNA was prepared from a single leaf with a single-step protocol (Thomson and Henry, 1995) or the DNeasy mini kit (Qiagen, Hilden, Germany). AFLP analysis was performed according to (Vos et al., 1995). A total of 210 F_2 individuals were scored. All primers and adaptors were obtained from Genset (Paris, France). Total gnomic DNA was digested by the restriction enzymes *Sac*I/*Mse*I. AFLP fingerprints were generated using

different primer combinations (markers of chromosome 1 and 4) with selectivity +2/+2. The DNA fingerprints were scored for presence or absence of bands.

Table 4: Detailed results of the linkage analysis for all individual samples on chromosome 1. L: Landsberg band, C: Columbia band, H: heterozygous band, /: no band, N.D.: not detected. Primers in the grey boxes are SSLP-primers, the others are AFLP-primers. M: mutant samples, WT: wild type samples out of the F2 population of *eer2* x Ler.

Primer		M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20
BC-19L		L	L	L	L	L	L	/	L	L	L	L	L	L	L	L	L	L	/	L
BA-1L			/	/	/	L	L	/	/	/	/	L	/	L	/	/	/	/	/	/
BC-21C/SM19_96.2			C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-20C/SM19_106.4			C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-10L			/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
nga280		C	C	C	C	C	C	C	C	H	C	C	C	C	H	C	C	C	C	C
nga111		H	C	H	H	C	C	H		H	H	H	C	H	H	H	H	H	C	C

	M21	M22	M24	M25	M26	M27	M28	M29	M30	M31	M32	M33	M34	M35	M36	M37	M38	M39	M40	M41
BC-19L	L	/	L	L	L	/	L	L	/	/	L	L	/	L	L	L	L	L	L	N.D.
BA-1L	/	/	/	L	L	/	L	/	/	/	L	/	/	L	/	L	/	L	L	/
BC-21C/SM19_96.2	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-20C/SM19_106.4	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-10L	/	/	/	/	/	/	/	/	/	/	/	/	/	L	/	/	/	/	/	/
nga280	C	C	H	C	C	C	C	C	C	C	C	C	C	H	C	C	C	C	C	C
nga111	C	C	C	H	C	H	C	C	C	H	H	L	C	H	C	C	C	C	C	C

	M42	M43	M44	M45	M46	M48	M49	M50	M51	M52	M53	M54	M55	M57	M58	M59	M60	M61	M62
BC-19L	/	L	L	/	L	L	/	/	L	L	/	/	L	L	/	L	L	L	L
BA-1L	/	L	/	/	L	/	/	/	/	L	/	/	/	/	/	L	/	L	/
BC-21C/SM19_96.2	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-20C/SM19_106.4	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-10L	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
nga280	C	C	C	H	C	C	C	C	C	C	C	H	C	C	C	C	C	C	C
nga111	C	C	C	H	H	C	H	C	C	C	L	H	L	C	H	C	H	C	C

	M63	M64	M65	M66	M67	M68	M69	M70	M71	M72	M73	M74	M75	M76	M82	M83	M84	M85	M86	M87
BC-19L	/	L	/	L	L	L	L	/	L	/	L	/	L	/	L	/	/	L	L	/
BA-1L	/	/	/	L	L	L	/	L	/	/	/	/	/	/	L	/	L	/	L	/
BC-21C/SM19_96.2	C	C	C	C	C	C	C	C	C	C	C	/	C	C	C	C	C	C	C	C
BC-20C/SM19_106.4	C	C	C	C	C	C	C	C	C	C	C	/	C	C	C	C	C	C	C	C
BC-10L	/	/	/	/	/	/	/	/	/	/	/	L	/	/	/	/	/	/	/	/
nga280	C	C	C	C	C	C	C	C	C	C	C	L	C	H	C	C	C	C	C	C
nga111	H	C	L	H	H	C	C	H	H	C	C	L	C	H	C	H	C	C	H	C

Table 4 - continued

	M88	M89	M90	M91	M92	M93	M95	M96	M97	M98	M99	M100	M101	M103	M104	M105	M106	M107	M108	M109
BC-19L	/	/	L	L	L	L	L	L	L	L	L	/	L	L	L	L	L	L	L	L
BA-1L	/	/	/	L	/	L	L	L	/	/	L	/	L	/	/	/	/	/	L	L
BC-21C/SM19_96.2	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-20C/SM19_106.4	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-10L	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
nga280	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
nga111	C	L	H	H	H	C	L	C	H	H	H	H	L	C	C	H	C	C	C	C

	M110	M111	M112	M113	M114	M115	M116	M117	M118	M119	M120	M121	M122	M123	M124	M125	M126	M127	M128	M129
BC-19L	L	/	L	/	L	L	L	L	/	/	L	L	L	L	L	L	L	L	/	L
BA-1L	/	/	/	L	L	/	/	/	/	/	/	L	L	/	L	/	/	/	/	/
BC-21C/SM19_96.2	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-20C/SM19_106.4	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-10L	/	/	/	/	/	/	/	/	/	/	/	/	L	/	/	/	/	/	/	/
nga280	C	C	N.D.	C	C	C	C	H	C	C	C	C	C	C	C	C	C	C	C	C
nga111	C	C	H	L	C	H	C	H	C	C	C	H	H	H	L	H	C	L	H	L

	M130	M131	M132	M133	M134	M135	M136	M137	M138	M139	M140	M141	M142	M143	M144	M145	M146	M147	M148	M149
BC-19L	L	L	/	L	L	L	L	/	/	L	L	/	/	L	L	/	/	L	L	L
BA-1L	/	/	/	/	/	/	/	/	/	L	/	/	/	/	/	/	/	/	/	/
BC-21C/SM19_96.2	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	/	C
BC-20C/SM19_106.4	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	/	C
BC-10L	/	/	/	/	/	/	L	/	/	/	/	/	/	/	/	/	/	/	/	/
nga280	C	C	C	C	C	H	C	C	C	C	C	C	C	C	C	N.D.	C	C	C	C
nga111	H	H	L	C	H	H	C	H	H	H	H	H	H	H	C	/	C	L	C	H

	M150	M151	M152	M153	M154	M155	M156	M157	M158	M159	M160	M161
BC-19L	L	L	N.D.	L	/	L	/	/	L	/	/	/
BA-1L	/	/	/	/	/	/	/	L	/	/	/	/
BC-21C/SM19_96.2	C	C	N.D.	C	C	C	C	C	C	C	C	C
BC-20C/SM19_106.4	C	C	N.D.	C	C	C	C	C	C	C	C	C
BC-10L	/	/	N.D.	/	/	/	/	/	/	/	/	/
nga280	C	C	C	C	C	C	C	C	C	C	C	C
nga111												

Table 4 - continued

	WT1	WT4	WT5	WT6	WT7	WT8	WT11	WT12	WT14	WT15	WT17	WT18	WT20	WT30	WT31	WT36	WT37	WT38	WT39	WT40
BC-19L	L	L	L	L	L	L	L	L	/	L	L	L	L	L	/	/	L	/	L	L
BC-21C/SM19_96.2	/	/	/	/	/	/	/	/	/	/	/	/	/	C	/	/	/	/	/	C
BC-20C/SM19_106.4	/	/	/	/	/	/	/	/	/	/	/	/	/	C	/	/	/	/	/	C
BC-10L	L	L	L	L	L	L	L	L			L	L	L	L	L	L	N.D.	L	/	L
nga 280	L	L	L	L	L	L	L	L	L	L	L	L	L	H	L	L	L	L	L	H

	WT41	WT43	WT44	WT45	WT46	WT47	WT48	WT49	WT50	WT51	WT52	WT53	WT54	WT55	WT56	WT57	WT60	WT61	WT62	WT65
BC-19L	L	/	N.D.	L	/	L	L	L	L	L	L	L	/	N.D.	L	L	L	L	/	N.D.
BC-21C/SM19_96.2	/	/	N.D.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	N.D.
BC-20C/SM19_106.4	/	/	N.D.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	N.D.
BC-10L	L	L	N.D.	L	L	L	L	L	L	L	L	L	L	N.D.	L	L	L	L	N.D.	N.D.
nga 280	L	L	L	L	L	L	H	L	L	L	L	L	L	L	L	L	L	L	L	L

	WT67	WT69	WT72	WT74	WT77	WT79	WT81	WT82	WT84	WT85	WT87	WT88	WT91	WT92	WT93	WT95	WT98	WT99	WT100	WT101
BC-19L	N.D.	L	L	L	L	/	L	L	/	N.D.	/	L	L	L	L	L	/	L	L	/
BC-21C/SM19_96.2	N.D.	/	/	/	/	/	/	/	C	N.D.	/	/	/	/	/	/	C	/	/	/
BC-20C/SM19_106.4	N.D.	/	/	/	/	/	/	/	C	N.D.	/	/	/	/	/	/	C	/	/	/
BC-10L	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
nga 280	L	L	L	L	L	L	L	L	L	L	L	L	/	/	/	/	/	/	/	/

	WT102
BC-19L	L
BC-21C/SM19_96.2	/
BC-20C/SM19_106.4	/
BC-10L	/
nga 280	/

Table 5: Detailed results of the linkage analysis for all individual samples on chromosome 4. L: Landsberg band, C: Columbia band, H: heterozygous band, /: no band, N.D.: not detected. Primers in the grey boxes are SSLP-primers, the others are AFLP-primers. M: mutant samples, WT: wild type samples out of the F2 population of *eer2* x Ler.

Primer		M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20
BC-11C/SM_186.9			C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-24L			/	/	/	/	/	/	/	/	/	/	/	/	L	/	/	/	/	/
Sac AC- Mse AA (226)			/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
nga8		C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
nga1111			C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BA-12L			/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
BC-4L			L	L	/	/	/	/	/	L	/	/	/	/	/	/	/	/	/	/
BC-17C/SM_144.9			/	/	/	/	/	/	/	/	/	/	/	/	L	/	/	/	/	/

	M21	M22	M24	M25	M26	M27	M28	M29	M30	M31	M32	M33	M34	M35	M36	M37	M38	M39	M40	M41
BC-11C/SM_186.9	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-24L	/	/	/	/	/	/	/	N.D.	/	/	/	/	/	/	/	/	/	/	/	/
Sac AC- Mse AA (226)	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
nga8	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
nga1111	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BA-12L	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
BC-4L	/	/	/	/	/	/	/	/	/	/	/	/	/	L	/	/	/	/	/	/
BC-17C/SM_144.9	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/

	M42	M43	M44	M45	M46	M48	M49	M50	M51	M52	M53	M54	M55	M57	M58	M59	M60	M61	M62	M63
BC-11C/SM_186.9	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-24L	/	/	/	/	/	/														C
Sac AC- Mse AA (226)	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
nga8	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
nga1111	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BA-12L	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
BC-4L	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	L	/	L	L	N.D.
BC-17C/SM_144.9	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	N.D.

	M64	M65	M66	M67	M68	M69	M70	M71	M72	M73	M74	M75	M76	M82	M83	M84	M85	M86	M87	M88
BC-11C/SM_186.9	C	C	C	C	C	C	C	C	C	C	L	C	C	C	C	C	N.D.	C	C	C
BC-24L		N.D.																		
Sac AC- Mse AA (226)	/		/	/	/	/	/	/	/	/	L	/	/	/	/	/	/	/	/	/
nga8	C	C	C	C	C	C	C	C	C	C	L	C	C	C	C	C	C	C	C	C
nga1111	C	C	C	C	C	C	C	C	C	C	L	C	C	C	C	C	C	C	C	C
BA-12L	/	/	/	/	/	/	/	/	/	/	L	/	/	/	/	/	/	/	/	/
BC-4L	L	/	L	/	/	/	/	/	/	/	L	L	/	L	/	/	/	L	/	/
BC-17C/SM_144.9	/	/	/	/	/	/	/	/	/	/	L	/	/	/	/	/	/	/	/	/

Table5 - continued

	M89	M90	M91	M92	M93	M95	M96	M97	M98	M99	M100	M101	M103	M104	M105	M106	M107	M108	M109	M110
BC-11C/SM_186.9	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-24L			C																	
Sac AC- Mse AA (226)	/	/	/	/	/	/	/	/	/	/	/	/								
nga8	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
nga1111	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BA-12L	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
BC-4L	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
BC-17C/SM_144.9	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/

	M111	M112	M113	M114	M115	M116	M117	M118	M119	M120	M121	M122	M123	M124	M125	M126	M127	M128	M129	M130
BC-11C/SM_186.9	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-24L	L	/	/	/	/	/	/	L	/	/	/	L	/							
Sac AC- Mse AA (226)																				
nga8	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
nga1111	C	C	C	C	C	C	C	C	C	C	C	C	C							
BA-12L	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
BC-4L	/	/	/	/	/	/	/	/	/	/	/	L	/	/	/	L	/	L	L	/
BC-17C/SM_144.9	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/

	M131	M132	M133	M134	M135	M136	M137	M138	M139	M140	M141	M142	M143	M144	M145	M146	M147	M148	M149	M150
BC-11C/SM_186.9	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
BC-24L																				
Sac AC- Mse AA (226)																				
nga8	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
nga1111																				
BA-12L	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
BC-4L	/	/	/	/	L	/	/	/	L	L	/	/	/	/	/	/	/	/	/	/
BC-17C/SM_144.9	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/

	M151	M152	M153	M154	M155	M156	M157	M158	M159	M160	M161
BC-11C/SM_186.9	C	N.D.	C	C	H	C	C	H	H	C	H
BC-24L											
Sac AC- Mse AA (226)											
nga8	C	C	C	C	C	C	C	C	C	C	C
nga1111											
BA-12L	/	/	/	/	/	/	/	/	/	/	/
BC-4L	/	N.D.	L	/	/	/	/	L	/	L	/
BC-17C/SM_144.9	/	N.D.	/	/	/	/	/	/	/	/	L

Table5 - continued

	WT1	WT4	WT5	WT6	WT7	WT8	WT11	WT12	WT14	WT15	WT17	WT18	WT20	WT30	WT31	WT36	WT37	WT38	WT39	WT40
BC-11C/SM_186.9	L	L	L	L	L	L	L	L	L	L	L	L	L	H	L	H	L	L	L	H
BC-24L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L		L	L	L
nga8	L	L	L	L	L	L	L	L	L	L	L	L	L	L	H	L	L	L	H	L
nga1111	L	L	L	L	L	L	L	L	L	L	L	L	L	H	L	H	L	L	L	L
BC-4L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
BC-17C/SM_144.9	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L		L	L	L	L

	WT41	WT43	WT44	WT45	WT46	WT47	WT48	WT49	WT50	WT51	WT52	WT53	WT54	WT55	WT56	WT57	WT60	WT61	WT62	WT65
BC-11C/SM_186.9	L	L	N.D.	L	L	L	L	L	L	L	L	L	L		L	L	L	L	L	N.D.
BC-24L	L	L		L	L	L	L	L	L	L	L	L	L		L	L	L	L		
nga8	L	L	L	L	L	L	H	L	L	L	L	L	L	L	L	L	L	L	L	L
nga1111	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L		
BC-4L	L		N.D.	L	L	L	L	L	L	L	L	L	L	N.D.	L	L	L	L	L	/
BC-17C/SM_144.9	L	L	L	L	L	L	L	L	L	L	L	L	L	N.D.	L	L	L	L	L	L

	WT67	WT69	WT72	WT74	WT77	WT79	WT81	WT82	WT84	WT85	WT87	WT88	WT91	WT92	WT93	WT95	WT98	WT99	WT100	WT101
BC-11C/SM_186.9	N.D.	L	L	L	L	L	L	L	L	N.D.	L	L	L	L	L	L	L	L	L	L
BC-24L																				
nga8	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
nga1111																				
BC-4L	N.D.	L	L	L	L	L	L	L	L	N.D.	L	L	L	L	L	L	L	L	L	L
BC-17C/SM_144.9	N.D.	L	L	L	L	L	L	L	L	N.D.	L	L	L	L	L	L	L	L	L	L

	WT102
BC-11C/SM_186.9	L
BC-24L	
nga8	L
nga1111	
BC-4L	L
BC-17C/SM_144.9	L

5.8 REFERENCES

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S., and Ecker, J.R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. Science **284,** 2148-2152.

Alonso, J.M., Stepanova, A.N., Solano, R., Wisman, E., Ferrari, S., Ausubel, F.M., and Ecker, J.R. (2003). Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in Arabidopsis. Proc Natl Acad Sci U S A **100,** 2992-2997.

Belouchi, A., Kwan, T., and Gros, P. (1997). Cloning and characterization of the OsNramp family from Oryza sativa, a new family of membrane proteins possibly implicated in the transport of metal ions. Plant Mol Biol **33,** 1085-1092.

Bleecker, A.B. (1999). Ethylene perception and signaling: an evolutionary perspective. Trends Plant Sci **4,** 269-274.

Bleecker, A.B., Estelle, M.A., Somerville, C., and Kende, H. (1988). Insensitivity to ethylene conferred by a dominant mutation in Arabidopsis thaliana seedlings. Science **241,** 1086-1089.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R. (1997). Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell **89,** 1133-1144.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc Natl Acad Sci U S A **81,** 1991-1995.

Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S., and McCourt, P. (1996). A protein farnesyl transferase involved in abscisic acid signal transduction in Arabidopsis. Science **273,** 1239-1241.

De Greef, J., M., D.P., and F, D.W. (1976). Gas chromatographic determination of ethylene in large air volumes at the fractional parts-per-billion level. Anal Chem 48: 3841 **48,** 38-41.

De Paepe, A., Vandenbussche, F., Arickx, I., Lootens, P., Vandecasteele, P., Van Montagu, M., and Van Der Straeten, D. (1999). Role of ethylene in senescence-associated programmed cell death in Arabidopsis thaliana. Archives of Physiology and Biochemistry 107, B7, 1999.

Delhaize, E. (1996). A metal-accumulator mutant of Arabidopsis thaliana. Plant Physiol **111,** 849-855.

Grbic, V., and Bleecker, A.B. (1995). Ethylene stimulates the timing of leaf senescence in Arabidopsis. Plant J. **8,** 595-602.

Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M.L., and Eide, D. (1998). Identification of a family of zinc transporter genes from Arabidopsis that respond to zinc deficiency. Proc Natl Acad Sci U S A **95,** 7220-7224.

Guzman, P., and Ecker, J.R. (1990). Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell **2,** 513-523.

Hensel, L.L., Grbic, V., Baumgarten, D.A., and Bleecker, A.B. (1993). Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in arabidopsis. Plant Cell **5,** 553-564.

Hirayama, T., Kieber, J.J., Hirayama, N., Kogan, M., Guzman, P., Nourizadeh, S., Alonso, J.M., Dailey, W.P., Dancis, A., and Ecker, J.R. (1999). RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. Cell **97,** 383-393.

Hua, J., and Meyerowitz, E.M. (1998). Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. Cell **94,** 261-271.

Hua, J., Chang, C., Sun, Q., and Meyerowitz, E.M. (1995). Ethylene insensitivity conferred by Arabidopsis ERS gene. Science **269,** 1712-1714.

Hua, J., Sakai, H., Nourizadeh, S., Chen, Q.G., Bleecker, A.B., Ecker, J.R., and Meyerowitz, E.M. (1998). EIN4 and ERS2 are members of the putative ethylene receptor gene family in Arabidopsis. Plant Cell **10,** 1321-1332.

Itzhaki, H., Maxson, J.M., and Woodson, W.R. (1994). An ethylene-responsive enhancer element is involved in the senescence-related expression of the carnation glutathione-S-transferase (GST1) gene. Proc Natl Acad Sci U S A **91,** 8925-8929.

Jing, H.C., Sturre, M.J., Hille, J., and Dijkwel, P.P. (2002). Arabidopsis onset of leaf death mutants identify a regulatory pathway controlling leaf senescence. Plant J **32,** 51-63.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R. (1993). CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases. Cell **72,** 427-441.

Korshunova, Y.O., Eide, D., Clark, W.G., Guerinot, M.L., and Pakrasi, H.B. (1999). The IRT1 protein from Arabidopsis thaliana is a metal transporter with a broad substrate range. Plant Mol Biol **40,** 37-44.

Koyama, T., Kitajima, S., and Sato, F. (2001). Expression of PR-5d and ERF genes in cultured tobacco cells and their NaCl stress-response. Biosci Biotechnol Biochem **65,** 1270-1273.

Kubo, M., and Kakimoto, T. (2000). The Cytokinin-hypersensitive genes of Arabidopsis negatively regulate the cytokinin-signaling pathway for cell division and chloroplast development. Plant J **23,** 385-394.

Lamoureux, G.L., and Rusness, D.G. (1993). Glutathione in the metabolism and detoxification of xenobiotics in plants. (SPB Academic Publishing).

Larsen, P.B., and Chang, C. (2001). The Arabidopsis eer1 Mutant Has Enhanced Ethylene Responses in the Hypocotyl and Stem. Plant Physiol **125,** 1061-1073.

Larsen, P.B., and Cancel, J.D. (2003). Enhanced ethylene responsiveness in the Arabidopsis eer1 mutant results from a loss-of-function mutation in the protein phosphatase 2A A regulatory subunit, RCN1. Plant J **34,** 709-718.

Lehman, A., Black, R., and Ecker, J.R. (1996). HOOKLESS1, an ethylene response gene, is required for differential cell elongation in the Arabidopsis hypocotyl. Cell **85,** 183-194.

Lincoln, J.E., and Fischer, R.L. (1988). Diverse mechanisms for the regulation of ethylene-inducible gene expression. Mol Gen Genet **212,** 71-75.

Lohman, K.N., Gan, S., John, M.C., and Amasino, R.M. (1994). Molecular analysis of natural leaf senescence in Arabidopsis thaliana. Physiol. Plant. **92,** 322-328.

Lootens, P., and Vandecasteele, P. (2000). A cheap chlorophyll a fluorescence imaging system. Photosynthetica **38,** 53-56.

Macnair, M.R., Bert, V., Huitson, S.B., Saumitou-Laprade, P., and Petit, D. (1999). Zinc tolerance and hyperaccumulation are genetically independent characters. Proc R Soc Lond B Biol Sci **266,** 2175-2179.

Oh, S.A., Park, J.H., Lee, G.I., Paek, K.H., Park, S.K., and Nam, H.G. (1997). Identification of three genetic loci controlling leaf senescence in Arabidopsis thaliana. Plant J **12,** 527-535.

Ouaked, F., Rozhon, W., Lecourieux, D., and Hirt, H. (2003). A MAPK pathway mediates ethylene signaling in plants. Embo J **22,** 1282-1288.

Rodrigues-Pousada, R.A., De Rycke, R., Dedonder, A., Van Caeneghem, W., Engler, G., Van Montagu, M., and Van Der Straeten, D. (1993). The Arabidopsis 1-Aminocyclopropane-1-Carboxylate Synthase Gene 1 Is Expressed during Early Development. Plant Cell **5,** 897-911.

Rodriguez, F.I., Esch, J.J., Hall, A.E., Binder, B.M., Schaller, G.E., and Bleecker, A.B. (1999). A copper cofactor for the ethylene receptor ETR1 from Arabidopsis. Science **283,** 996-998.

Roman, G., Lubarsky, B., Kieber, J.J., Rothenberg, M., and Ecker, J.R. (1995). Genetic analysis of ethylene signal transduction in Arabidopsis thaliana: five novel mutant loci integrated into a stress response pathway. Genetics **139,** 1393-1409.

Sakai, H., Hua, J., Chen, Q.G., Chang, C., Medrano, L.J., Bleecker, A.B., and Meyerowitz, E.M. (1998). ETR2 is an ETR1-like gene involved in ethylene signaling in Arabidopsis. Proc Natl Acad Sci U S A **95,** 5812-5817.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. 2nd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, NY.

Sidler, M., Hassa, P., Hasan, S., Ringli, C., and Dudler, R. (1998). Involvement of an ABC transporter in a developmental pathway regulating hypocotyl cell elongation in the light. Plant Cell **10,** 1623-1636.

Smalle, J., Haegman, M., Kurepa, J., Van Montagu, M., and Van Der Straeten, D. (1997). Ethylene can stimulate Arabidopsis hypocotyl elongation in the light. Proc Natl Acad Sci U S A **94,** 2756-2761.

Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R. (1998). Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev **12,** 3703-3714.

Thomine, S., Wang, R., Ward, J.M., Crawford, N.M., and Schroeder, J.I. (2000). Cadmium and iron transport by members of a plant metal transporter family in Arabidopsis with homology to Nramp genes. Proc Natl Acad Sci U S A **97,** 4991-4996.

Thomine, S., Lelievre, F., Debarbieux, E., Schroeder, J.I., and Barbier-Brygoo, H. (2003). AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. Plant J **34,** 685-695.

Thomson, D., and Henry, R. (1995). Single-step protocol for preparation of plant tissue for analysis by PCR. Biotechniques **19,** 394-397, 400.

Van Der Straeten, D., Djudzman, A., Van Caeneghem, W., Smalle, J., and Van Montagu, M. (1993). Genetic and Physiological Analysis of a New Locus in Arabidopsis That Confers Resistance to 1-Aminocyclopropane-1-Carboxylic Acid and Ethylene and Specifically Affects the Ethylene Signal Transduction Pathway. Plant Physiol **102,** 401-408.

Van Der Straeten, D., Smalle, J., Bertrand, S., De Paepe, A., Vandenbussche, F., Arickx, I., Haegman, M., Van Caeneghem, W., Fostier E., and Van Montagu, M. (1999). Ethylene signaling in Arabidopis: more players in the game. Abstract presented at the Belgium-Netherlands Phytohormone Meeting 1999 "Plantengroeiregulatoren: metabolisme, functie en werkingsmechanismen", Antwerpen (Belgium), January 8, 1999.

Vandenbussche, F., Smalle, J., Le, J., Saibo, N.J., De Paepe, A., Chaerle, L., Tietz, O., Smets, R., Laarhoven, L.J., Harren, F.J., Van Onckelen, H., Palme, K., Verbelen, J.P., and Van Der Straeten, D. (2003). The Arabidopsis mutant alh1 illustrates a cross talk between ethylene and auxin. Plant Physiol **131,** 1228-1238.

Vogel, J.P., Schuerman, P., Woeste, K., Brandstatter, I., and Kieber, J.J. (1998). Isolation and characterization of Arabidopsis mutants defective in the induction of ethylene biosynthesis by cytokinin. Genetics **149,** 417-427.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and et al. (1995). AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res **23,** 4407-4414.

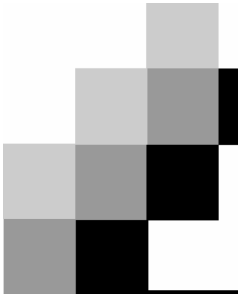
Woeste, K.E., and Kieber, J.J. (2000). A strong loss-of-function mutation in RAN1 results in constitutive activation of the ethylene response pathway as well as a rosette-lethal phenotype. Plant Cell **12,** 443-455.

Woo, H.R., Goh, C.H., Park, J.H., Teyssendier de la Serve, B., Kim, J.H., Park, Y.I., and Nam, H.G. (2002). Extended leaf longevity in the ore4-1 mutant of Arabidopsis with a reduced expression of a plastid ribosomal protein gene. Plant J **31,** 331-340.

Yang, S.F., and Hoffman, N.E. (1984). Ethylene biosynthesis and its regulation in higher plants. Ann. Rev. Plant. Physiol. **35,** 155-189.

Zegzouti, H., Jones, B., Frasse, P., Marty, C., Maitre, B., Latch, A., Pech, J.C., and Bouzayen, M. (1999). Ethylene-regulated gene expression in tomato fruit: characterization of novel ethylene-responsive and ripening-related genes isolated by differential display. Plant J **18,** 589-600.

Zhou, J., and Goldsbrough, P.B. (1993). An Arabidopsis gene with homology to glutathione S-transferases is regulated by ethylene. Plant Mol Biol **22,** 517-523.



Chapter 6

Screening and preliminary analysis of novel hypersensitive ethylene mutants

Chapter 6: Screening and preliminary analysis of novel hypersensitive ethylene mutants

Annelies De Paepe, Mira Haegman, Els Fostier, Cedric De Smet, Angelo Titeca and Dominique Van Der Straeten

6.1 SCREENING FOR HYPERSENSITIVE ETHYLENE MUTANTS

Mapping of the *eer2* locus was hampered by linkage on two different chromosomes. Therefore, we decided to look for *eer2*-alleles by performing a new screening for hypersensitive mutants. To that end, a collection of 90.000 EMS-mutagenized seeds was used. From an EMS-mutagenized population, mutants displaying either a point mutation or a small insertion or deletion are expected. This is in contrast with the first screening for which FN-mutagenized seeds were used, resulting normally in mutants with larger deletions. Besides *eer2*, this first screening also yielded two additional FN-lines displaying longer hypocotyls, which were further investigated. The screening of an EMS-mutagenized population resulted in new ethylene hypersensitive mutants, extending the class of enhanced ethylene response mutants. Out of the 90.000 EMS-mutagenized seeds, 222 seedlings showed longer hypocotyls on LNM+1μM ACC compared to the wild type upon a first screening. These lines were rescreened and this resulted in 62 candidates for hypersensitive mutants. All candidates were further tested in dose-response experiments to investigate their hypersensitive behaviour. From this analysis 18 mutants displayed an enhanced response to ACC. It should be mentioned here that these mutants are not strictly hypersensitive mutants because hypersensitivity implies more responsiveness to ACC at all tested concentrations as is the case for *eer2.* For some of the isolated mutants, enhanced responsiveness is seen in the presence of 1μM ACC, which is significantly higher than the wild type, but hypocotyls are as long as the wild type on the media containing the higher ACC concentrations. Due to the biological variability between the different individual experiments, the results are displayed separately; each graph is accompanied by the wild type and *eer2* controls and in two of the four experiments also by an ethylene insensitive control (Fig. 1). By this screening, our collection of mutants displaying enhanced ethylene responses was expanded; these additional mutants could be *eer2*-alleles, alleles of previously characterized components or novel factors in ethylene biosynthesis or signalling.

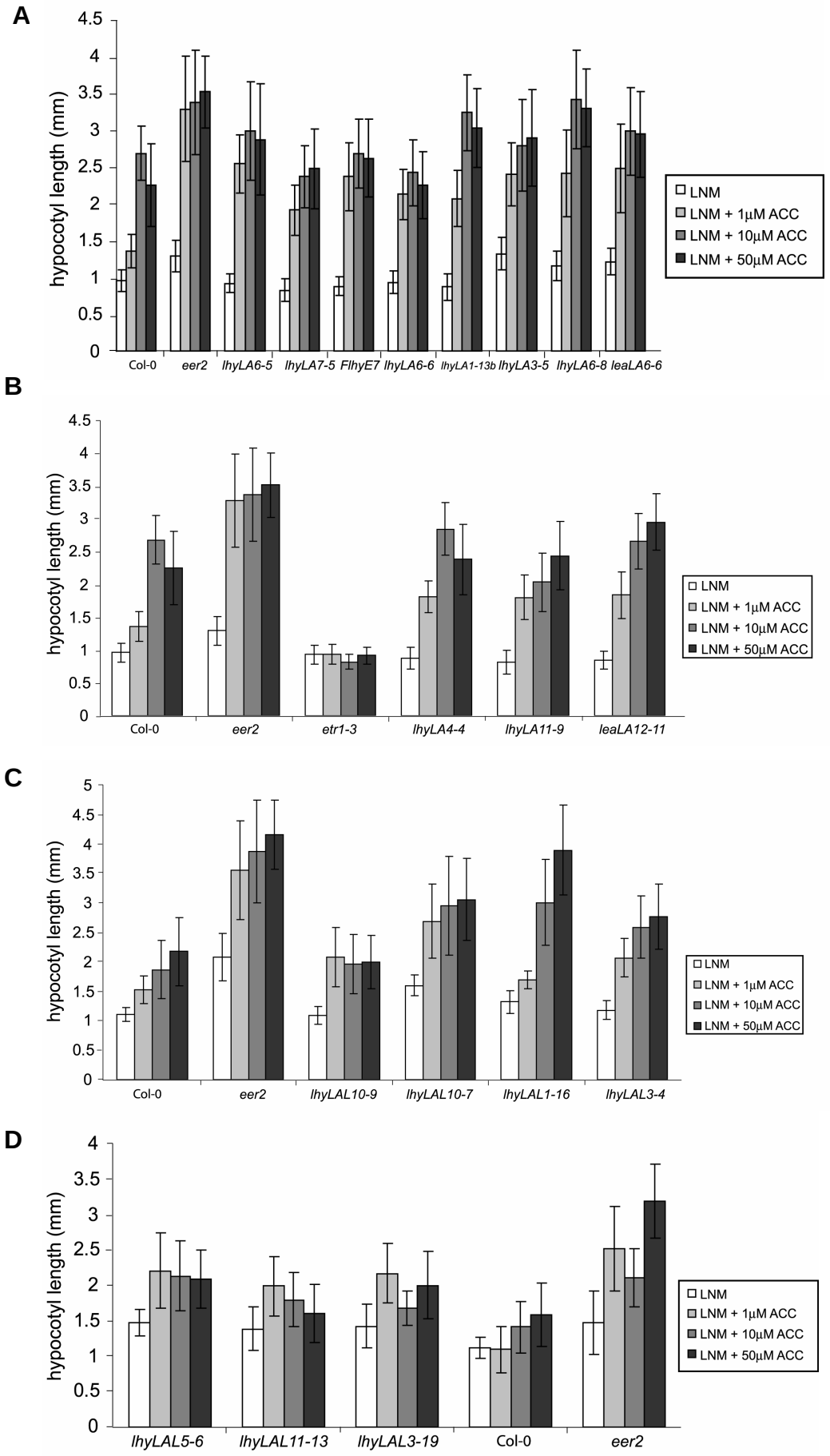


Figure 1: Effect of ACC on hypocotyl elongation in the light. A, B, C, D: Seedlings of wild type (Col-0), *eer2*, *etr1-3,* and 18 candidate mutants were grown for 8 days on LNM medium in the light supplemented with ACC in a range of concentrations. Data are mean ± SD (n>30).

6.2 PRELIMINARY RESULTS ON THE NEW SET OF MUTANTS

6.2.1 Hypocotyl response test in the dark

On a subset of our group of the candidate *eer*-mutants, a dose-response test in the dark was performed (Fig. 2). Four out of seven tested mutants, lhyLA6-5, lhyLA6-6, lhyLA7-5 and FlhyE7 were more sensitive to 1µM ACC. Thus, for these mutants the response in the light and the dark is similar, meaning a more pronounced response to ACC compared to wild type. LhyLA6-6 also displayed a higher inhibition of hypocotyls growth on the media containing higher concentrations of ACC. In contrast, lhyLA1-13b displayed a less pronounced response on the medium supplemented with 1µM ACC; lhyLA6-8 was less sensitive to ACC on all tested concentrations. The same response was stronger in *eer2*, as described before (chapter 5). LeaLA6-6 displayed a normal response. Due to the phenotypic differences in the dark between the mutants, we concluded that *eer2* is probably not allelic to lhyLA6-5, lhyLA6-6, lhyLA7-5, FlhyE7, and leaLA6-6. In contrast, lhyLA6-8 and lhyLA1-13b, could be alleles of *eer2*.

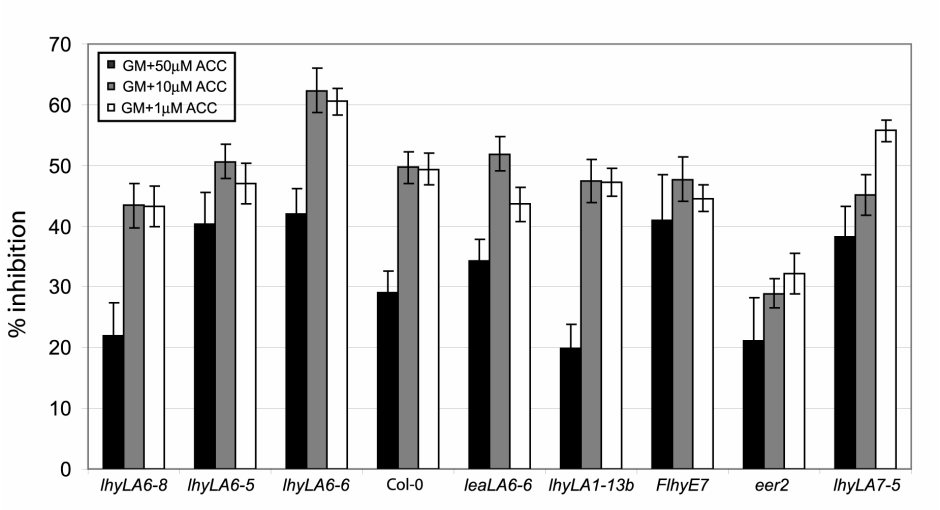


Figure 2: Effect of ACC on hypocotyl growth inhibition in the dark. Seedlings of wild type (Col-0), *eer2*, and candidate mutants were grown for 5 days on GM medium in the dark supplemented with ACC in a range of concentrations. Data are mean ± SD (n>30).

6.2.2 Backcrosses with Col-O

In order to obtain an idea of the strength of the mutation (dominance, semi-dominance, or recessivity), the mutants were backcrossed to Col-0. If the mutant phenotype is present in the F1-generation, the mutant confers a dominant trait. To that end, the hypocotyl length on LNM + 1µM ACC was measured. At present, only for five mutants (lhyLA6-5, lhyLA1-13b, lhyLAL10-9, and FlhyE7) hypocotyl measurements were performed.

Hypocotyl elongation test on LNM + 1µM ACC:

- Col-0 x lhyLA6-5, lhyLA1-13b, lhyLAL10-9, lhyLA6-6: Col-0 phenotype
- Col-0 x FlhyE7: intermediate phenotype between Col-0 and FlhyE7

From these results, we can conclude that lhyLA6-5, lhyLA1-13b, LhyLAL10-9 and LhyLA6-6 are recessive mutants, whereas FlhyE7 could be semi-dominant. However, we have to consider that only a few seeds were available. Final conclusions can only be made by mapping the mutant and further phenotypic characterization.

6.2.3 Allelism-tests

Some of the novel *eer*-mutants were crossed with *eer2*. Since four candidate alleles were causing recessive mutations, it was possible to select *eer2*-alleles from the phenotype of the F1 progeny. If both mutants are alleles, the *eer2*-phenotype can not be complemented by the other mutation. In this test also other candidate mutants were analysed, for which no data concerning the strength of the mutation is available yet. For this analysis, the two most obvious phenotypes of *eer2* were tested: the longer hypocotyls on LNM + 1µM ACC and the de-greening of rosettes on GM after 3 weeks of growth.

De-greening test on GM:

- *eer2* x lhyLA6-5, lhyLA7-5, FlhyE7, LhyLA1-13b, leaLA6-6, lhyLAL10-9 and lhyLAL10-7: no de-greening
- *eer2* x lhyLA6-8: *eer2*-phenotype (= de-greening)

Hypocotyl elongation test on LNM + 1µM ACC:

- *eer2* x lhyLA3-5 , lhyLAL3-4, lhyLA6-8, lhyLA7-5, lhyLA6-5: WT-phenotype
- *eer2* x lhyLAL10-2, FlhyE7, lhyLAL10-9, lhyLAL5-6, lhyLAL11-13: *eer2*-phenotype

Based on these results, we infer that some mutants are putative alleles of *eer2*. However, due to several reasons, we cannot be sure of this:

- in the F1-generation only a few seeds (1-6) were available for measuring, this is too low to yield statistically significant results
- if the tested mutant confers a dominant trait, then the observed phenotype in the F1-generation is due to the dominant mutation

6.2.4 Preliminary mapping results

All novel *eer* mutants were also crossed with Ler to generate a mapping population. Nine mutants, (lhyLA6-8, lhyLA1-13b, lhyLA7-5, lhyLA10-7, FlhyE7, lhyLA6-5, leaLA6-6, lhyLAL5-6, lhyLA6-6) were chosen to map. The choice was based on the facility of scoring for mutant and wild type phenotypes in the F2 generation. Eleven SSLP-markers, covering the five chromosomes of *Arabidopsis*, were tested. These results are presented in table 1. For all tested mutants except lhyLAL5-6, the markers on chromosome 2 demonstrated the closest linkage with the mutations. None of the tested mutants showed linkage with nga 8, which was found to be situated close to the *eer2* mutation. Thus, we could conclude that none of the tested mutants are allelic to *eer2*. However, because almost all tested mutants displayed linkage with chromosome 2 markers, these mutants could possibly be alleles of one another. Therefore, allelism-tests are on the program for the future.

6.3 FUTURE WORK

The screening for additional mutants displaying an enhanced response to ACC for hypocotyl elongation on LNM resulted in a number of new interesting mutants. Mainly the screening, isolation and confirmation of the phenotype were done during this Phd thesis. All additional experiments are in a preliminary stage and therefore no final conclusions concerning the mutations could yet be made.

Until now, the mapping populations contain only a small number of selected mutants and wild type samples; therefore more individual samples are needed for each mapping population to be able to find the locus of interest. Moreover, some phenotypes of selected mutants should be reconfirmed.

Considering the observation of the common linkage on chromosome 2, a number of allelism-test should be performed between the respective mutants.

Table 1: Overview of mapping results of novel *eer* mutants

	Chromosome 1				Chromosome 2							
	nga59		nga280		nga1126		nga361		nga168		AthBIO2	
	Number of tested individuals	Result	Number of tested individuals	Result	Number of tested individuals	Result	Number of tested individuals	Result	Number of tested individuals	Result	Number of tested individuals	Result
Ler x lhyLA6-8	\	\	38	n	9	l	9	l	38	\	9	l
Ler x lhyLA1-13b	18	\	46	n	\	\	\	\	18	l	\	\
Ler x lhyLA7-5	15	n	59	n	31	l	31	l	74	\	31	l
Ler x lhyLAL10-7	\	\	36	\	20	l	20	l	31	l	20	l
Ler x FlhyE7	10	n	21	n	19	l	19	l	38	n	19	\
Ler x lhyLA6-5	16	n	9	n	17	l	17	l	33	l	17	\
Ler x leaLA6-6	36	n	26	n	18	l	18	l	58	n	18	l
Ler x lhyLAL5-6	\	\	21	n	\	\	\	\	18	n	\	\
Ler x lhyLA6-6	53	n	19	n	\	\	\	\	38	l	\	\

	Chromosome 3				Chromosome 4		Chromosome 5			
	nga162		nga6		nga8		nga139		AthCTR1	
	Number of tested individuals	Result	Number of tested individuals	Result	Number of tested individuals	Result	Number of tested individuals	Result	Number of tested individuals	Result
Ler x lhyLA6-8	38	n	38	n	38	n	38	n	38	n
Ler x lhyLA1-13b	6	n	18	n	41	n	6	l	6	\
Ler x lhyLA7-5	68	n	15	n	36	n	5	n	5	n
Ler x lhyLAL10-7	\	\	\	\	14	n	\	\	\	\
Ler x FlhyE7	40	n	36	\	21	n	21	n	21	n
Ler x lhyLA6-5	7	n	33	n	22	\	7	n	7	n
Ler x leaLA6-6	33	n	28	n	73	n	33	n	33	n
Ler x lhyLAL5-6	\	\	\	\	28	n	\	\	\	\
Ler x lhyLA6-6	9	n	38	n	48	n	9	n	9	n

n = no linkage
l = linkage
\ = not tested yet

6.4 EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Approximately 90.000 ethylmethanesulphonate (EMS) mutagenised M2 Columbia seeds (Lehle seeds, Round Rock, TX) were screened. Columbia (Col-0) was also purchased from Lehle seeds The wild type Ler-0 (Landsberg *erecta*) as well as the ethylene response mutant *etr1-3* originated from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University. Conditions of the growth chamber and greenhouse were 22°C and 60% relative humidity with white fluorescent light (75 µmol/m^2.s) and long day conditions (16 h light/8 h dark).

Media and treatments

The *Arabidopsis* seedlings were grown under sterile conditions as described in Smalle et al. (Smalle et al., 1997). The rich medium used was GM (Growth Medium) supplemented with 0.5 g/L of MES (Roman and Ecker, 1995). ACC (1-amino-cyclopropane-1-carboxylic acid) was obtained from Sigma-Aldrich (St. Louis, MO). The hormone solution was added to the medium after filter sterilization. Plates were stored at 4°C in the dark for 2 days and then put in a growth chamber.

Biometric analysis

Measurements of hypocotyl length of light grown seedlings were done as in Smalle et al. (1997) using a Stemi SV 11 microscope equipped with a graduated ocular (Zeiss, Jena, Germany).

Linkage analysis

Mapping of the new *eer*-mutants was performed with simple sequence length polymorphism markers (Bell and Ecker, 1994). All mutants were crossed to Ler. The F_2 population was scored for mutant and wild type plants based on the following characteristics: the increase in hypocotyls length on ACC, phenotype on soil. Per F_2 individual, DNA was prepared from a single leaf with a single-step protocol l(Edwards et al., 1991).

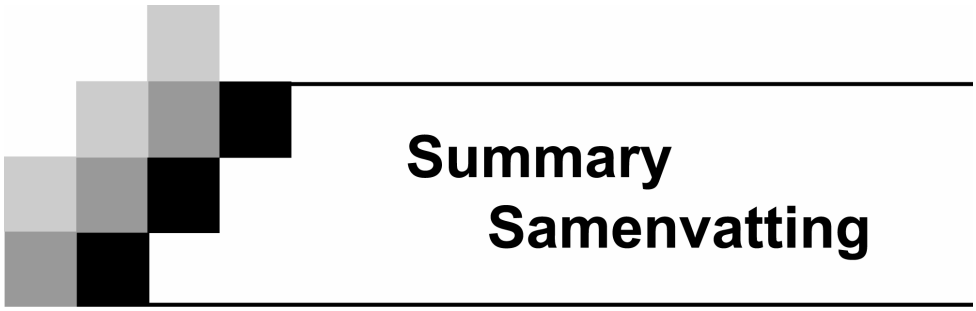
6.5 REFERENCES

Bell, C.J., and Ecker, J.R. (1994). Assignment of 30 microsatellite loci to the linkage map of Arabidopsis. Genomics **19,** 137-144.

Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res **19,** 1349.

Roman, G., and Ecker, J.R. (1995). Genetic analysis of a seedling stress response to ethylene in Arabidopsis. Philos Trans R Soc Lond B Biol Sci **350,** 75-81.

Smalle, J., Haegman, M., Kurepa, J., Van Montagu, M., and Van Der Straeten, D. (1997). Ethylene can stimulate Arabidopsis hypocotyl elongation in the light. Proc Natl Acad Sci U S A **94,** 2756-2761.



Summary
Samenvatting

Analysis of ethylene signalling in *Arabidopsis* using transcript profiling and novel ethylene-response mutants.

Ethylene is a simple two-carbon gaseous molecule with profound effects on plant growth and development, including promotion of phenomena such as the seedling triple response, response to pathogen attack, fruit ripening, and tissue senescence (Yang and Hoffman, 1984). Although a tremendous progress has been made elucidating the mechanisms of synthesis, perception, and transduction of the ethylene signal (described in **Chapter 1**), little is known about the genes that are transcriptionally regulated in response to ethylene. In an attempt to identify novel genes implicated in ethylene response, we screened for early ethylene-responsive genes in *Arabidopsis* using a cDNA-AFLP-transcript profiling approach and microarrays.

The identification of early ethylene-regulated genes is a strategy to gain further understanding of the molecular mechanisms of ethylene action. **Chapter 2** describes the results of a pilot cDNA-AFLP transcript profiling experiment in which the transcriptome was followed in a detailed time course upon treatment of the plants with ethylene. In order to isolate truly ethylene-responsive genes, the ethylene-insensitive mutant *ein2-1* and the constitutive ethylene-response mutant *ctr1-1* were analyzed in parallel. The expression pattern of about 1200 genes was monitored of which approximately 4% was differentially expressed. Sequence analysis revealed the identity of 45 of these genes. Database searches revealed that 77 % of the isolated tags were highly homologous to genes with known function, and 23 % of the tags matched a cDNA or genomic sequence with a yet unknown function. Interestingly, we identified transcripts displaying a temporal increase in mRNA levels during the earliest time points of ethylene treatment. The genes identified in this study belong to diverse functional groups, including transcriptional and post-transcriptional regulators, protein degradation, cell wall metabolism, signal transduction components, defence response, metabolic processes, hormone signalling and finally photosynthesis. Furthermore, analysis of ethylene responsiveness of 22 genes involved in the ubiquitin degradation pathway revealed that at least 4 genes, UBP4, UBP23, UBP27, and a putative UBC are induced by the hormone. Therefore, we demonstrated that proteolytic degradation is part of ethylene signalling. In conclusion, this study provided new insights into the interaction between the ethylene signal transduction pathway and other pathways.

In **Chapter 3** the physiological analysis of SALK lines carrying an insertion in ethylene-modulated genes is described. Altogether, for 6 out of 40 genes, identified in the cDNA-

AFLP profiling, a role in ethylene signalling was confirmed by functional analysis of their respective T-DNA insertion line. Of particular interest are the results of the mutant with its insertion in the gene encoding the MADS box protein AGL14, which displayed less sensitive responses to ACC in two of the three ethylene-response tests that were performed.

The genome-wide approach by microarray analysis described in **Chapter 4** extended the collection of previously identified early ethylene-regulated genes. Out of the 6008 genes present on the chip, 214 genes showed significant differential expression during the time course in wild type as compared to the insensitive mutant *ein2-1*. The cluster analysis and functional classification allowed us to determine classes of co-ordinately regulated genes under ethylene control. In particular, a large number of genes involved in cell rescue, disease and defence mechanisms were identified as early ethylene-regulated genes. Interestingly, these genes were mainly down-regulated by ethylene which is contradictory to the known positive role of ethylene in defence. Based on these observations, it can be concluded that ethylene is probably an active component differentially controlling the damage process depending on the infection phase. Furthermore, the data provide insight into early regulatory steps of ethylene signalling and ethylene-regulated transcription and protein fate. The results also reveal novel understanding of the integration of the ethylene pathway with other signals. Of particular interest is the overlap between ethylene response and responses to ABA, sugar and auxin. In addition, an evaluation and a comparative analysis between both transcript profiling experiments are illustrated in this chapter.

Another way to gain more insight in ethylene signalling is by isolating ethylene-response mutants. The screening method was based on the ethylene-induced hypocotyl elongation response of nutrient-deficient seedlings in the light (Smalle et al., 1997). In **Chapter 5** the analysis of a novel ethylene-related mutant, *eer2*, a second member of a new class of ethylene response mutants, the **e**nhanced **e**thylene **r**esponse mutants, is described. Besides its hypersensitive response in ethylene-induced hypocotyl elongation, *eer2* also displays enhanced response at the molecular level and at later stages of development in the light. Furthermore, *eer2* is also affected in its senescence pattern and its phenotype depends on the nutritional status of the growth medium. Therefore, EER2 may be either a factor involved in the cross-talk between ethylene and senescence or chloroplast development or a protein involved in accumulation or translocation of metals. In addition, this study demonstrated that novel screens help to identify new ethylene-related loci apart from those implicated at the etiolated seedling stage. By further screening, our collection of mutants displaying enhanced ethylene responses was expanded, as described in **Chapter**

6. Mainly the screening, isolation and confirmation of the phenotype were performed during this PhD thesis. These additional mutants could be possible *eer2*-alleles, alleles of previously characterized components or new interesting features in ethylene biosynthesis or signalling. None of the tested mutants showed linkage with the SSLP-marker, which was found to be situated close to the *eer2* mutation. Thus, we could conclude that none of the tested mutants are allelic to *eer2.* However, because all tested mutants displayed linkage with chromosome 2 markers, these mutants could possibly be alleles of one another. Therefore, allelism-tests are on the program for the future.

References

Smalle, J., Haegman, M., Kurepa, J., Van Montagu, M., and Van Der Straeten, D. (1997). Ethylene can stimulate Arabidopsis hypocotyl elongation in the light. Proc Natl Acad Sci U S A **94,** 2756-2761.

Yang, S.F., and Hoffman, N.E. (1984). Ethylene biosynthesis and its regulation in higher plants. Ann. Rev. Plant. Physiol. **35,** 155-189.

Onderzoek van ethyleen respons in *Arabidopsis* aan de hand van transcript profiling en mutant analyse.

Ethyleen is een eenvoudig twee koolstof atomen bevattend gas met talrijke effecten in plantengroei en ontwikkeling. Deze zijn ondermeer de inductie van de triple respons in zaailingen, de reactie op pathogen-infectie, vruchtrijping en veroudering (Yang en Hoffmann, 1994). Hoewel er een enorme vooruitgang is gemaakt in de kennis van de biosynthese, de perceptie en de transductie van ethyleen (beschreven in **hoofdstuk 1**), is er weinig geweten over de transcriptionele regulatie van ethyleen respons genen. Om nieuwe genen in de ethyleen respons te identificeren, hebben we via de cDNA-transcript profiling techniek en via microarray analyse vroege ethyleen-gereguleerde genen geïndentificeerd in *Arabidopsis*. De identificatie van vroege ethyleen-gereguleerde genen laat toe meer inzicht te verwerven in het moleculaire mechanisme van de werking van ethyleen.

Hoofdstuk 2 beschrijft de resultaten van het cDNA transcript profiling experiment waarin het transcriptoom opgevolgd werd gedurende een gedetailleerd tijdsverloop in aanwezigheid van ethyleen. Met als doel alleen ethyleen-gereguleerde genen op te pikken, werden de ethyleen-ongevoelige mutant *ein2-1* en de constitutieve ethyleen-respons mutant *ctr1-1* in parallel onderzocht. De expressieprofielen van ongeveer 1200 transcript fragmenten werden beoordeeld en daarvan was ongeveer 4% differentieel geëxpresseerd. Via sequeneren en het opzoeken in de databanken van de fragmenten werd 45 genen geïdentificeerd. Van de geïsoleerde transcript fragmenten waren 77% sterk homoloog met genen met een reeds gekende functie; 23% kwam overeen met een cDNA of genomische sequentie waarvan de functie nog niet gekend is. Een interessant resultaat van deze studie is dat een tijdelijke inductie in mRNA niveau werd geobserveerd gedurende de kortste ethyleen behandelingen. De geïdentificeerde genen uit dit piloot experiment maken deel uit van verschillende functionele klassen. Deze zijn ondermeer transcriptionele en post-transcriptionele regulatie, eiwit degradatie, celwand metabolisme, signaal transductie, defensie, metabolische processen, hormoon signaaloverdracht en fotosynthese. Verder werden 22 genen betrokken in de ubiquitine degradatie pathway onderzocht op hun responsiviteit op ethyleen. Uit dit onderzoek waren er tenminste 4 genen, namelijk *UBP4*, *UBP23*, *UBP27*, en een vermoedelijk *UBC* die door ethyleen werden geïnduceerd. We hebben dus aangetoond dat eiwit degradatie een deel uitmaakt van ethyleen signaaloverdracht. Uit dit alles, kunnen we besluiten dat deze studie tot

nieuwe inzichten leidde in de interactie van ethyleen signaaloverdracht met andere signaaloverdrachtswegen.

In **hoofdstuk 3** werd de fysiologische analyse van SALK-lijnen met een insertie in ethyleen-gemoduleerde genen beschreven. In het totaal werd voor 6 van 40 genen, opgepikt via cDNA-AFLP, een rol in ethyleen signaaloverdracht bevestigd via de functionele analyse van de respectievelijke insertie mutant. In het bijzonder was er de mutant met een insertie in het gen dat codeert voor een MADS-box eiwit AGL14 die in twee van de drie ethyleen-respons testen een minder gevoelige respons op ACC (1-amino-cyclopropaan-1-carbonzuur) vertoonde.

De genoom-wijde aanpak via microarray analyse breidde de collectie van eerder geïdentificeerde vroege ethyleen-gereguleerde genen uit. Dit experiment is beschreven in **hoofdstuk 4**. Van de 6008 genen op de chip, toonden 214 genen een significante differentiële expressie in het wild type ten opzichte van de ongevoelige mutant *ein2-1* gedurende de onderzochte tijdspanne. Via cluster analyse en functionele classificatie was het mogelijk klassen van genen die coördinatief ethyleen-gereguleerd zijn te bepalen. Een interessante observatie was dat een groot aantal genen die betrokken zijn in defensie- en verdedigingsmechanismen van de plant neer-gereguleerd waren door ethyleen. Dit resultaat is tegengesteld tot wat eerder was beschreven voor de rol van ethyleen in defensie, namelijk een positieve regulatie van defensie-genen. Door deze observaties was het mogelijk te besluiten dat ethyleen wellicht een actieve component is in de controle van de schade aan de plant op verschillende niveaus en dat ethyleen een verschillende rol speelt in defensie in functie van de tijd. Bovendien leidden de data ook tot meer inzicht in de vroege stappen van ethyleensignaaloverdracht, ethyleen-gereguleerde transcriptie en eiwit regulatie. Verder werden ook nieuwe inzichten verworven in de interactie van ethyleen met andere signalen. In het bijzonder werd een overlap aangetoond tussen ethyleen responsen en responsen van ABA, suiker, en auxine.

In dit hoofdstuk werd ook een evaluatie en vergelijking tussen de twee gebruikte transcript profiling methodes uiteengezet.

Een andere manier om meer inzicht te verwerven in ethyleen signaaloverdracht is via de isolatie van ethyleen respons mutanten. De gebruikte screeningsmethode was gebaseerd op de inductie van elongatie in het hypocotyl door ethyleen in het licht (Smalle et al., 1997). In **hoofdstuk 5** werd de analyse van de ethyleen mutant *eer2* beschreven. Deze mutant is een tweede lid van een nieuwe klasse van ethyleen respons mutanten, die gekarakteriseerd worden door versterkte ethyleen responsen. Naast de hypersensitieve respons van *eer2* in hypocotyl elongatie, vertoont deze mutant ook versterkte responsen

op het moleculair niveau en op latere stadia van de ontwikkeling in het licht. Bovendien vertoont *eer2* een ander verouderingspatroon en is het fenotype afhankelijk van de nutriënten in het medium. Uit dit alles blijkt dat EER2 kan betrokken zijn in de interactie tussen ethyleen en veroudering, chloroplast ontwikkeling of opname of transport van mineralen. Daarnaast is ook aangetoond dat nieuwe screeningsmethodes kunnen bijdragen in de identificatie van nieuwe ethyleen-gerelateerde factoren naast degene die nodig zijn tijdens etiolering. Verdere screening leidde tot identificatie van een collectie van mutanten met een versterkte ethyleen respons. Dit is beschreven in **hoofdstuk 6**. Tijdens dit doctoraat werden de fenotypes eveneens bevestigd in een volgende generatie wat verder onderzoek verantwoordt. Deze additionele mutanten kunnen mogelijks allelen zijn van *eer2*, allelen van eerder gekarakteriseerde componenten of nieuwe factoren in ethyleen biosynthese of -signaaloverdracht. Geen enkele van de geteste mutanten toonden koppeling met de SSLP-merker die heel dicht bij de *eer2* mutatie ligt. We kunnen dus besluiten dat geen enkele van de geteste mutanten allelisch is met *eer2*. Daarentegen toonden deze mutanten wel koppeling met merkers op chromosoom 2, waaruit we kunnen afleiden dat deze mutanten mogelijks alllelisch zijn. Daarom staan allelie-testen op het programma voor de toekomst.

References

Smalle, J., Haegman, M., Kurepa, J., Van Montagu, M., and Van Der Straeten, D. (1997). Ethylene can stimulate Arabidopsis hypocotyl elongation in the light. Proc Natl Acad Sci U S A **94,** 2756-2761.

Yang, S.F., and Hoffman, N.E. (1984). Ethylene biosynthesis and its regulation in higher plants. Ann. Rev. Plant. Physiol. **35,** 155-189.

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“Those wide horizons science offers never let you forget how little you know and understand….”